Summer 1982

Electroimmunoassay a New Competitive Protein Binding Assay Using Antibody Selective Electrodes

George Raymond Connell
Old Dominion University

ELECTROIMMUNOASSAY: A NEW COMPETITIVE PROTEIN BINDING ASSAY USING ANTIBODY SELECTIVE ELECTRODES

by

George Raymond Connell
B.S. May 1979, Atlantic Christian College

A Thesis Submitted to the Faculty of
Old Dominion University in Partial Fullfilment of the
Requirements for the Degree of

MASTER OF SCIENCE

CHEMISTRY

OLD DOMINION UNIVERSITY
August, 1982

Approved by:

Kenton M. Sanders (Co-director)

Roy L. Williams (Co-director)

Billy J. Upchurch

Thomas O. Sitz

ABSTRACT

ELECTROIMMUNOASSAY: A NEW COMPETITIVE PROTEIN BINDING ASSAY USING ANTIBODY SELECTIVE ELECTRODES

George Raymond Connell

Old Dominion University

Directors: Dr. Roy L. Williams and Dr. Kenton M. Sanders

The purpose of this study was to utilize the concept of antibody selective electrodes to develope a new competitive protein binding assay. This is a new concept and a potentially important analytical technique because it combines the advantages of RIA (sensitivity and selectivity) with the advantages of electrical assays (speed and low cost). A conjugate of PGE_2 and dibenzo-18-crown-6 (a cation selective ionophore) was synthesized, and incorporated into a plastic membrane. The ionophore conjugate increased the selectivity of a polyvinyl chloride (PVC) membrane to monovalent cations. Transmembrane potential was altered by anti-PGE_2 antisera in a concentration dependent manner, whereas non-immune serum was ineffective. The effect of anti-PGE_2 antibodies on membrane potential was reversible. Therefore, this antibody electrode was considered as a possible tool to assay prostaglandins. An effective antibody concentration was chosen and PGE_2 was added to the antibody solution. "Free" PGE_2 competed with the membrane bound PGE_2 and reduced the effect of the antibody as a function of "free" PGE_2 concentration. Standard curves were generated that could be used to quantitate unknown quantities of PGE_2 in solution. The assay system demonstrated specificity toward prostaglandins of the E series. Mechanisms possibly responsible for the effect of antibodies on membrane potential are discussed.

To my parents, George and Margaret Connell.

ACKNOWLEDGEMENTS

The author wishes to acknowledge Dr. Kenton Sanders for providing insite, optimism and an environment conducive to learning and personal growth. I also thank Kent for his help in preparation of this thesis.

I am also grateful to:

-Dr. Roy Williams for his help during the course of this project.
-Drs. Thomas Sitz and Billy Upchurch for their contribution as members of my research commitee.
-Dr. Frank Scully for performing NMR analysis.
-Dr. Robert Cotter for performing fast atom bombardment mass spectal analysis.
-Dr. Nelson Publicover for helpful suggestions regarding the preparation of this manuscript and presentation of data.

In addition, I thank my fellow students and co-workers for their support during the course of this project.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The development of practical means of measuring minute samples of biologically active compounds is important to endocrinologists, clinical chemists, physiologists and industrial biologists. Appropriate assays should be fast, accurate, and inexpensive.

Mass Spectrometry

A very sensitive analytical instrument, the mass spectrometer, is able to detect extremely small amounts of organic compounds in biological samples, and when used in conjunction with a gas chromatograph to separate different compounds in a mixture, it is of great value(1). The main drawbacks to the adaptation of the mass spectrometer for general use in biological laboratories are i) the cost of the instrument, ii) many of the compounds of interest to biologists are compounds with high molecular weight composed of repetitive subunits making fragmentation analysis difficult; iii) samples which are not volatile must be derivatized before analysis can be achieved. Fast atom

bombardment (FAB), currently the state of the art technique, allows the detection of molecular ions of high molecular weight compounds (2). This method was used to determine the molecular ion of a compound I synthesized for this project and provided information unattainable by other methods.

High performance liquid chromatography

High performance liquid chromatography (HPLC) can also be used to measure small quantities of compounds(3). The HPLC consists of a hydraulic pump, column and detector. The separation of compounds is achieved on the column through which the mixture of compounds is passed along with suitable solvents. As with other types of liquid chromatography the chemical aspects of the compounds separated influence their association with either the column material or the solvents. The advantages of HPLC are: i) a refined HPLC system can distinguish between compounds with related structure, and ii) it is easily adapted to automated analysis. The disadvantages include: i) the apparatus is expensive to operate, ii) samples may require chemical modification to attach detectable labels, iii) standards must be used in

order to compare their retention times with the retention times of unknown samples.

Bioassay

Bioassay is another method for determining the concentrations of biologically active materials. Living tissue is used to quantitate the concentration of a biologically active substance by measuring a specific biological response. Standard curves are generated by recording the biological response as a function of standard concentrations of the compound to be assayed. Then the tissue is treated with unknown concentrations of the same compound and responses are used to "solve" the function that best models the standard curve(4). Drawbacks to this type of assay are: i) other agents in the unknown sample may have biological activity which may lead to spurious results; ii) during the course of an assay the tissue may become less responsive; iii) tachyphylaxis or desensitization of the receptors of the assay tissue may give values from the standard curve that may underestimate actual concentrations in the tested solutions.

Radioimmunoassay

The most widely used method for detection of small quantities of biologically active compounds is radioimmunoassay (RIA). It is applicable to many analytical problems because it combines both sensitivity (small quantities can be detected) and selectivity (compounds of similar structure can be distinguished). Radioimmunoassay is a type of competitive protein binding assay. In these assays radioactively labelled molecules [*A] and non labelled molecules [A] of identical structure except for the label are allowed to react with a specific binding protein until equilibrium is reached. Ideally the binding protein has equal and high affinity for [*A] and [A]. Therefore [A] and [*A] compete for the binding sites of the protein. At equilibrium bound and free [*A] are separated from each other, and either bound or free are counted, giving a quantitative measurment of the concentration of bound [*A].

In order to measure unknown samples that contain [A], a calibration plot is generated by plotting bound [*A] as a function of [A] concentration. To a series of tubes containing a fixed concentration of [*A] and binding

protein, various concentrations of [A] are added. The free, unlabelled [A] competes with [*A] for binding sites. The more [A] present, the less [*A] is bound to the protein. Unknown quantities of [A] in biological samples can be determined by adding a known amount of antibody and [*A] to the unknown biological sample and counting the bound counts. The bound [*A] values can be used to solve the standard curve and determine what amount of [A] must be present to yield the experimentally determined degree of [*A] binding(5).

The sensitivety and selectivety of an RIA is dependent on the quality of the antibody. A good antibody must have high affinity and specificity toward the compound to be assayed. The affinity is important in determining the concentration range over which the assay is sensitive. Antibodies with high affinity can be saturated with low concentrations of the compound. Therefore, labelled compound can be displaced by low concentrations of the unlabelled compound to be measured.

In most cases it is not possible to produce antibodies to compounds of molecular weight less than 5,000-10,000 unless they are covalently conjugated with immunogenic

molecules(6). The compound of low molecular weight for which an antibody is desired is called a hapten. The hapten is coupled to an immunogenic compound to produce an antigenic hapten-carrier conjugate.

The design of antigenic hapten-carrier conjugates involves the selection of a suitable immunogenic molecule to attach to the hapten. It is also necessary to determine the point of attachment required to yield a conjugate that will produce the best possible antibody. The immunogenic compound is usually a protein. The proteins usually chosen for conjugation are ribonuclease A MW 14,000, tobacco mosaic viral protein MW 17,500, lysozyme MW 14,000, egg albumin MW 40,000, serum albumin MW 70,000, thyroglobulin MW 669,000. All of these molecules are excellent antigens (chemically complex macromolecues that are soluble in but foreign to the body fluids of the animal to to be immunized)(7). Generally, the greater the molecular weight the more potent the antigen. The larger the molecule, the greater the chance that it will have chemical complexity, which will be recognized as a antigenic determinant.

The immunogenic conjugate is injected into an animal to promote antibody formation. At the optimal time after the

immunogenic challenge, blood is collected from the animal and the immune serum is harvested. The usefulness of antisera is evaluated by determining the titer(number of immune molecules) and the affinity of the immune binding sites. Titer is determined by titrating antibody dilutions with a fixed concentration of immunogen or hapten.

The antibody affinity constant is determined by a Scatchard analysis, which plots the relationship between bound and unbound antigen at equilibrium. The higher the concentration ratio of bound antigen the higher the affininty of the antibody. In addition antibodies with high affinity have high specificity. The affinity is expressed as the equilibrium constant, which is a measurement of the strength of the antigen antibody interaction (8). An antisera with high titer and high affinity is most useful in performing RIA because i) dilute sera can be used for the assay which reduces the concentration of non-specific binding sites, ii) sera with high affinity can be saturated with low concentrations of labelled anitigen, iii) low concentration of unlabelled antigen can displace the bound labelled antigen which allows the detection of extremely low concentrations of antigen.

While RIA is an extremely useful as a method to assay large numbers of samples, there are a few problems: i) the precedure for a specific assay may be time consuming ii) this method is also dependent on the use of radioactively labelled compounds which are expensive and require special handling, iii) in many cases the use of RIA is not practical unless a large number of samples are routinely processed because each time an assay is run a calibration plot must be generated, iv) equipment to perform RIA, while standard in research labs, is expensive to buy.

Electrical assay

The use of an electrical method for the analysis of biologically active compounds has several advantages; i) the speed of electrical analysis, ii) in most cases electrical methods are inexpensive, requiring little equipment and few standard reagents, iii) sample preparation is minimized because most electrodes are able to detect the desired analyte in mixtures.

All electrical methods must function by measuring charge movement. Often this is achieved by using ion

selective electrodes which contain membranes that are selectively permiable to a single ion or a class of ions. If the ion selectivity of the membrane can be coupled in some way to a specific chemical reaction involving the biological compound to be assayed, then it is sometimes possible to measure concentrations of the compound as a function of transmembrane potential.

A membrane can be made selectively permiable to a particular ion by incorporating an ionophore into the membrane. Ionophores are organic compounds that are able to bind ions. The general structural aspects of these molecules is a hydrophilic interior surrounded by a hydrophobic exterior. Thus, many ionophores can transport ions across hydrophobic membranes. In many cases the selectivity of the ions which are bound by these compounds is remarkable. They are used as the active components of ion selective electodes. The different classes of cation specific antibiotics are cyclodepsipeptides, macrotetrolides, nigericin-type antibiotics, and macrocyclic polyethers. The following is a short description of these ion carriers.

Valinomycin is a member of the class cyclodepsipep-

tide. It was the first ionophore to be extensively studied, and forms complexes with potassium. Nonactin, a member of the actins, is able to form complexes with potassium. Monesin is a member of the nigericin class of ionophores. The above ionophores are all naturally occuring antibiotics which are isolated from plants. Their similarities include: i) each has a composition of subunits linked by ester linkages, amide linkages, and carbon-carbon bonds, ii) they have cyclic stuctures, iii) they are able to form complexes with cations, iv) the complexes formed are generally thermodynamically stable, v) they undergo stereochemical changes upon complexation, vi) they have a hydrophilic interior which allows them to complex the cations, this interior includes oxygens with non-bonding electrons which interact with cations vii) the relative hydrophobic nature of their exteriors allows the transport of ions through organophilic mediums i.e. PVC membranes. Their differences include: i) the number and type of subunits, ii) the ion most favorably complexed, iii) monesin is a carboxylic acid which coordinates cations in its anionic state. Dibenzo-18-crown-6, a member of the macrocyclic polyethers, is also able to complex cations. It

differs from the others in that: i) it is not a natural product, but the result of chemical synthesis, ii) its cation binding ring is composed of repeating ethyl units linked by ether linkages, iii) it does not undergo radical conformational changes upon complexation. The structure of dibenzo-18-crown-6 is shown in figure 1 (9). This figure includes several different views of the compound; the chemical formula, the conformation of the uncomplexed molecule, and the structure of the molecule complexed with $NaBr \cdot 2H_2O$.

Gas sensitive electrodes have been used in combination with enzyme systems to determine the concentrations of compounds that are able to be enzymatically converted and release a measurable gas. An example of this is the use of an ammonium gas sensitive electrode in combination with a slice of kidney tissue with an active urea cycle i.e. (the ability to deaminate glutamine and release ammonium). Ammonium is detected by the ammonium sensitive electrode(10). Other gas electrode-enzyme combinations have been used to analyze urea, glucose, arginine, phenylalanine, penicillin, creatinine, uric acid, adenosine monophosphate, lactate dehydrogenase, urease, cholinesterase and many other

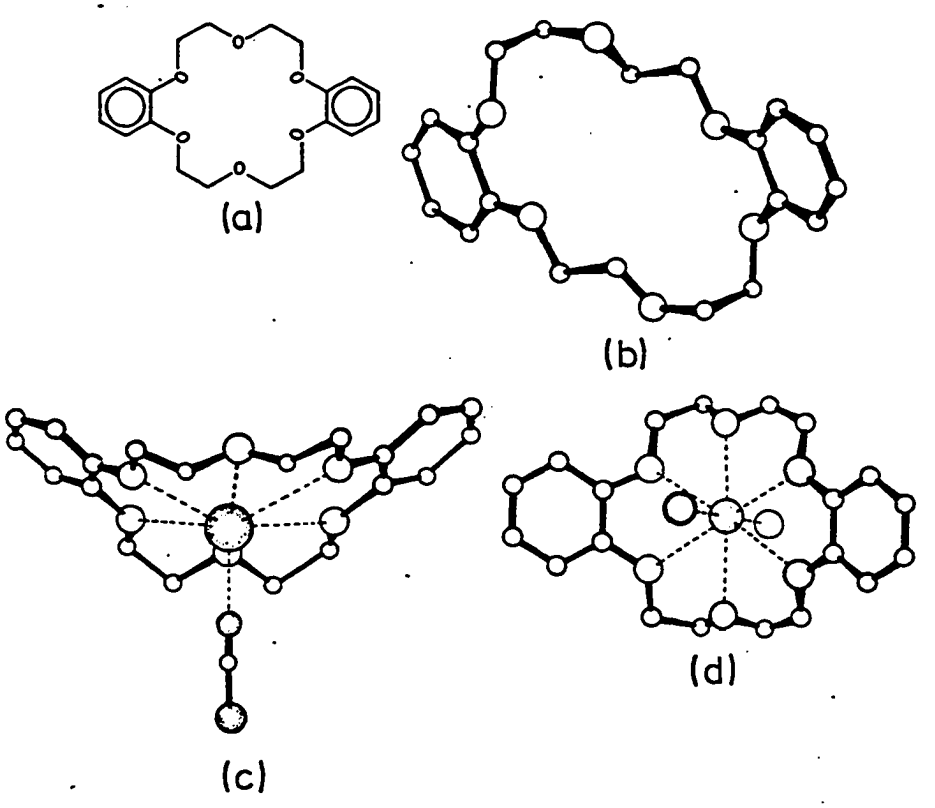


Figure 1. The structure of dibenzo-18-crown-6 (a) chemical structure, (b) confomation of the uncomplexed molecule, (c) RbNCS complex, (d) NaBr $2H_2O$ complex. (9)

enzymatically convertible compounds (11). The advantages of these systems are their high selectivity and reliability. Their disadvantages include: i) they are rather delicate systems because enzyme activity must be preserved, ii) electrodes that employ enzymes in living tissues require fresh tissues, iii) they are only useful for analyzing compounds specifically converted to measurable metabolites by readily available enzymes.

Another bioanalytical electrode is the antibody electrode developed by Solsky et al which uses ionophore immunogen conjugates in a PVC membrane to measure specific antibody concentration(12). The electrode, developed by Solsky, is able to detect anti-dinitrophenol antibody. The ionophore immunogen conjugate is composed of dibenzo-18-crown-6(a cation selective ionophore) with dinitrophenol covalently bound to its benzene rings.

Dibenzo-18-crown-6 is used in this electrode because the structure of dibenzo-18-crown-6 is ideal for chemical modification. Substitutions can be made to the benzene rings to introduce new functions into the compound, such as the addition of amine groups which are added by first nitration of the dibenzo-18-crown-6, followed by reduction

of these groups to amines. Once amine groups are added, substituents can be attached to the molecule through these groups. In contrast, antibiotic type ionophores are not open to modification because their structure is in most cases lacking any areas to which selective additions to their basic structure can be made, outside of the heterocyclic ring.

The combination of immunogen and ionophore results in molecule able to selectively transport ions through the PVC membrane, the selectivity is effected by the interaction of antibody(13). One important advantage of this technique over the enzyme electrodes is, as stated above, the enzyme electrode is only applicable to compounds for which an enzyme is availble to convert the compound of interest and release a measurable compound. However, antibodies can be developed for almost any compound, therefore antibody electrodes could be developed for almost any antigen-antibody system.

The work done by Solsky demonstrated that antibody electrodes could be developed for anti-dinitrophenol antibodies. He suggested that similar electrodes could be developed for any hapten-antibody combination. The

mechanism suggested by Solsky for antibody selectivity is an interaction between membrane-phase hapten and solution-phase antibody. This is possible because serum from non-immunized animals did not effect membrane potential. The specificity of the antibody-hapten interaction could be exploited to assay hapten ccncentration, because addition of solution-phase hapten to antibody samples should yield a competition between membrane and solution phase hapten for the antibody. This might reduce the potentiometric response to the antibody in a manner dependent on the concentration of solution phase hapten. Therefore, the concentration of solution-phase hapten might be quantified by the degree of reduction in the antibody effect. Standard curves of antibody effect vs. free hapten concentration could be used to assay unknown concentration of hapten in solution. The development of an assay for prostaglandin E_2 (PGE_2) was persued because its measurment by this method would provide a useful tool for the investigation of the biological activity of PGE_2.

To test this hypothesis an anti-PGE_2 antibody electrode was developed by synthesizing a PGE_2-dibenzo-18-crown-6 conjugate. The conjugate was dissolved in a PVC membrane

and placed into an electrode body. Once the antibody selectivity of this membrane was established the possibility of using this electrode in a competitive protein binding assay was explored. At a given antibody concentration PGE_2 was added to the antibody samples. The "free" PGE_2 competed with membrane bound PGE_2 for antibody and reduced the potentiometric response of antibody as a function of "free" PGE_2 concentration. Therefore, standard curves were generated by plotting membrane potential vs. "free" PGE_2 concentration.

METHODS

Synthesis

A conjugate of PGE_2 and dibenzo-18-crown-6 was synthesized. The conjugate was formed using an ethyl chloroformate reaction (14) to couple the acid function of the PGE_2 molecule, with amine groups synthetically placed on the crown ether. PGE_2-*trans* diamide of dibenzo-18-crown-6 is a new compound and its synthesis presented some problems because: i) PGE_2 is expensive, ii) *trans* diamino dibenzo-18-crown-6 has limited solubility, and iii) the PGE_2 mixed anhydride is rather unstable.

To learn efficient synthetic procedures several reactions with model compounds were performed to practice the technique and learn the problems associated with each step of the synthesis. The mixed anhydride reaction was performed first with linoleic acid and palmitic acid as substitutes for PGE_2. These two fatty acids were chosen because of their structural similarities to PGE_2. Large quantities of the mixed anhydride of these fatty acids were synthesized and identified by IR and NMR spectroscopy. The

amide synthesis was tested by substituting p-anisidine for trans-diamino-dibenzo-18-crown-6, and coupling palmitic acid anhydride with p-anisidine. After the model syntheses were mastered, the PGE_2-trans diamide of dibenzo-18-crown-6 was easily synthesized.

Reagents

The reagents used for the synthesis of the PGE_2-trans-diamide of dibenzo-18-crown-6 , membrane preparation and the model studies which were certified ACS grade or better included: i) dibenzo-18-crown-6 (PCR Research Chemical Inc., Gainsville, Fla.), ii) prostaglandin E_2(PGE_2) (Upjohn), iii) linoleic acid (Sigma Chemical Co.,St.Louis,MO), iv) palmitic acid (Sigma), v) triethylamine (Mallinkrodt, Inc., St.Louis, Mo), vi) ethyl chloroformate (Eastman Organics, Rochester,NY) , vii) polyvinyl chloride, high MW,(Eastman Organics), viii) dibutyl sebacate (Eastman Organics), ix) tetrahydrofuran (THF) (Aldrich Chemical Co., Milwaukee, Wis.), x) Chloroform (Mallinckrodt), xi) N,N-dimethyformamide (Aldrich), xii) acetonitrile (Mallinckrodt), xiii) Raney nickel #28 (W.R.

Grace Inc.), xiv) nitric acid (Mallinckrodt), xv) glacial acetic acid (Mallinckrodt), xvi) diethyl ether, anhydrous (Mallincrodt).

Trans diamine of Dibenzo-18-crown-6

Synthesis of the trans-diamine of dibenzo-18-crown-6 was carried out in three steps: i) nitration of dibenzo-18-crown-6, ii) separation of the cis and trans dinitro-products, and iii) reduction to the trans-diamine. The nitration reaction was carried out as follows. A one liter round bottom flask equipped with a stirrer, water cooled condenser, dropping funnel and a heating mantle was charged with 20 g (0.056 M) of dibenzo-18-crown-6 (figure A1), dissolved in 400 ml of chloroform, 300 ml of glacial acetic acid was then added to this solution. The nitrating solution, consisting 14 ml of nitric acid and 40 ml glacial acetic acid was prepared and added to the stirring mixture dropwise at a rate of 2 ml/min. After the entire nitrating solution was added the reaction was stirred for one hour without heat and then three hours at reflux. After three hours the product could be seen

as yellow crystals. The reaction was then filtered giving 8.56 g (0.0196 M) of crude trans dinitro product. The crude product was recrystalized from N,N-dimethylformamide (DMF) to give 7.48 g (0.0166 M) of yellow crystals with a melting point of 239-245°C (literature MP 237-246°C) (15). The product was identified by using IR spectroscopy(Figure A2). Nitro bands could be seen at 6.58 μ (1520 cm^{-1}) and 7.43 μ (1345 cm^{-1}). The IR spectra of trans-dinitro-dibenzo-18-crown-6 is shown in the appendix.(Figure A2)

The cis dinitro dibenzo-18-crown-6 precipitated from the mother liquor after cooling for one hour. The yield of cis product was 6.4 g (0.0142 M) and the melting point was 209-234°C. (literature MP 206-232° C.) (15). The IR spectrum of the cis dinitro product is shown in the appendix(Figure A2). Only the trans dinitro product was carried through the rest of the synthetic procedure.

The trans diamine was prepared by the catalytic reduction of the trans dinitro product in a Parr hydrogenator. Into a 500 ml shaker bottle, 2.0 g (0.0044 M) of trans dinitro-dibenzo-18-crown-6 was placed and 200 ml of DMF added together with 2.0 g of Grace Raney nickel

Raney nickel #28. The mixture was shaken at 40 psi of hydrogen for one hour. After hydrogenation the catalyst was removed by filtration and the product was collected by first removing the DMF by vacuum distillation. The crude diamino product was purified by sublimation at 0.1 mm. Hg. and 240-260°C. After purification the product was a white crystalline solid which melted at 198-203°C.(literature MP 199-203°C) (15). This product was also identified by IR (Figure A3)and exhibited absorption bands at 3.04 μ (3289 cm^{-1}), 2.97 μ (3367 cm^{-1}), 6.17 μ (1621 cm^{-1}) charactoristic of the NH_2. The NMR spectra of the dinitro compound and the diamine could not be obtained due to their insolubility.

Preparation of palmitic acid amide of anisidine

To demonstrate that a mixed anhydride could react with a primary aromatic amine, p-anisidine was condensed with the palmitic acid mixed anhydride (Figure A4). A solution of 0.256 g (1 mM) palmitic acid in 100 mL of ether was placed into a round bottom flask equipped with a magnetic stirrer, water cooled condenser and a drying tube. 0.101 g (1 mM) of

triethylamine was added and the mixture stirred for one hour. 0.108 g (1 mM) ethyl chloroformate was then added and the mixture was stirred for an additional hour. Triethylamine hydrochloride precipitated from the reaction mixture and was removed by filtration leaving 0.328 g (1 mM) of the palmitic acid mixed anhydride in solution, which was treated with 0.123 g (1 mM) of p-anisidine and the mixture was stirred for one hour. The product, a white precipitate, was isolated from the reaction mixture by filtration. The product was found to be relatively pure via TLC, and it's melting point was 104°C. The structure was confirmed by IR and NMR spectral analysis (Figure A5). The IR spectra showed charactoristic amide carbonyl peak at 6.06 μ (1650 cm^{-1}), NH-strech at 2.86 μ (3500 cm^{-1}) and NH bend at 6.17 μ (1620 cm^{-1}). The NMR spectrum integrated for 31 aliphatic protons at 1.1 ppm, 3 protons at 3.7 ppm from the methoxy group and 4 aromatic protons at 6.8 ppm.

The linoleic acid trans diamide of dibenzo-18-crown-6

A solution of 0.2804 g (1.0 mM) of linoleic acid in 100 ml of anhydrous ether was placed in a 200 ml round

bottom flask equipped with a magnetic stirrer, water cooled condenser and a drying tube. The solution was stirred and 0.1012 g (1.0 mM) of triethylamine was added and then stirred for one hour. 0.1085 g (1.0 mM) of ethyl chloroformate was then added and stirred for an additional hour. Due to the size of this reaction the precipitation of triethylamine hydrochloride is seen after a few minutes supporting the formation of the linoleic acid mixed anhydride in situ. The triethylamine hydrochloride was removed by filtration. The ether was removed and the condensation reaction was performed in acetonitrile.

In a separate flask, 0.195 g (0.05 mM) of diamino-dibenzo-18-crown-6 was dissolved in 100 ml of acetonitrile. Once the diamine was dissolved the solution of the mixed anhydride was added and stirred overnight. The derived product precipitated from the reaction mixture and was subsequently purified by recrystalization from chloroform/absolute ethanol and dried with anhydrous ether, the melting point was 174°C. The product was analized by IR and NMR spectra (Figure A6). The IR spectra showed charactoristic amide carbonyl streching at 6.06 μ (1650 cm^{-1}), NH strech at 2.90 μ (3500 cm^{-1}), and NH bend at 6.16

μ (1620 cm^{-1}). The elemental analysis calculated for linoleic acid trans-diamide of dibenzo-18-crown-6 is 73.47% Carbon, 9.47% Hydrogen, 3.06% nitrogen, 13.97% oxygen; found 73.89% Carbon, 9.43% Hydrogen, 3.00% nitrogen, 13.86% oxygen.

PGE_2 trans-diamide of dibenzo-18-crown-6

A mixture of 7.04 mg (0.02 mM) of PGE_2 in 15 ml of anhydrous ether was placed into a 50 ml round bottom flask equipped with a magnetic stirrer and a water cooled condenser 2.024 mg (0.02 mM) of triethyl amine was then added and the mixture was stirred for one hour. At this point 2.170 mg (0.02 mM) of ethylchloroformate was added, and the mixture was again stirred for one hour. After one hour the presence of triethylamine hydrochloride was indicated by the cloudiness of the solution. The triethylamine hydrochloride was removed from the reaction mixture by filtration, and the mixed anhydride (Figure 2) was retained in the ether solution. The diamine is not soluble in ether, so acetonitrile was used in the condensation reaction with the mixed anhydride.

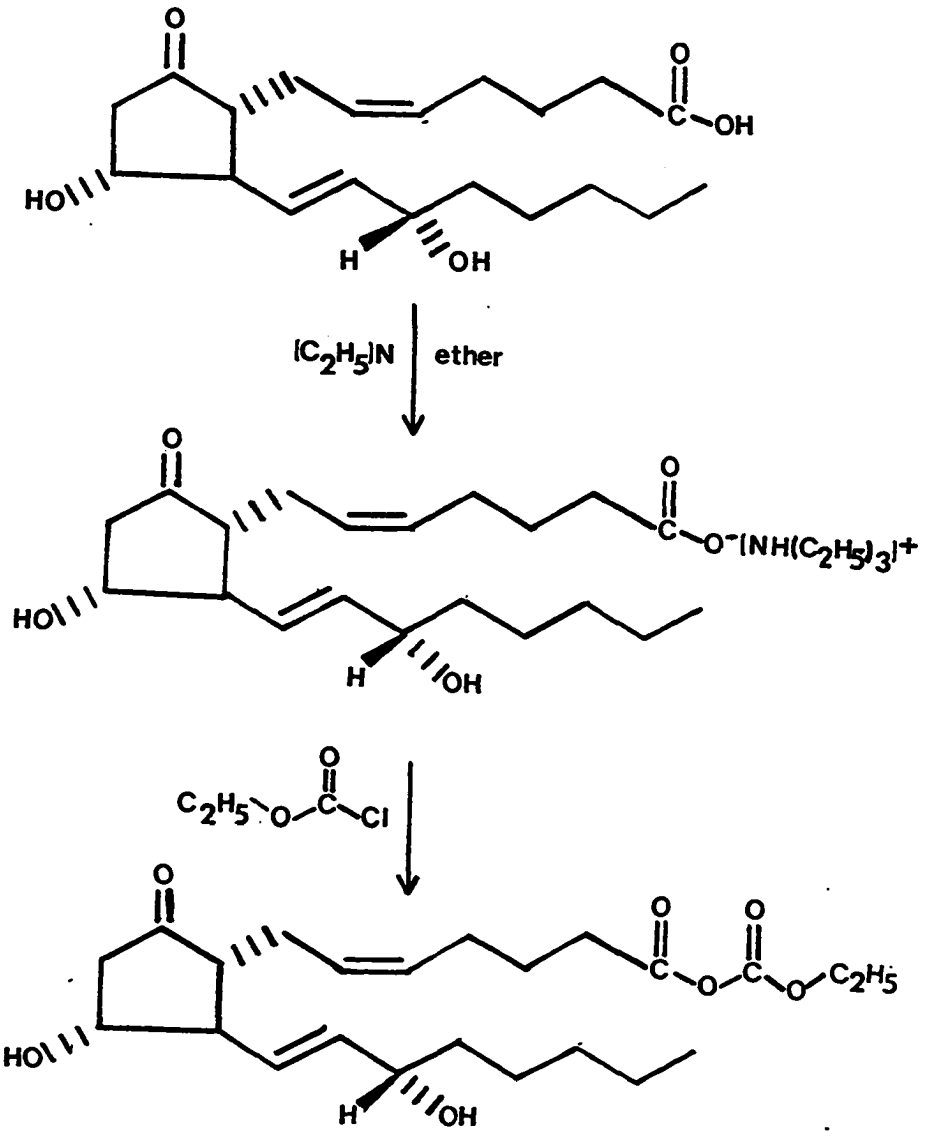


Figure 2. The synthetic scheme for the formation of PGE_2 mixed anhydride.

Therefore, the ether was removed under vacuum and was replaced with 20 ml of acetonitrile. In a seperate flask 3.900 mg (0.01 mM) diamino-dibenzo-18-crown-6 was dissolved in 20 ml of acetonitrile, slowly added to the solution of PGE_2 mixed anhydride and stirred for twelve hours. The condensation reaction which resulted in the formation of the PGE_2-*trans* diamide of dibenzo-18-crown-6 is summarized in Figure 3.

The acetonitrile was removed under vacuum and about 5 ml of anhydrous ether was added to dry and wash the crystals which coated the sides of the flask. The extremely small amount of product excluded the possibility of using normal recrystallization techniques so the crystals were purified by recrystallization in the flask. The product was purified by dissolving the crude material in about 1 ml of chloroform; an equal amount of absolute ethanol added and this mixture concentrated *in vacuo*. The chloroform was removed first which allowed the product to precipitate in the absolute ethanol. The ethanol was also removed, and the crystals were again dried with anhydrous ether. The product was then identified by NMR and mass spectral analysis (Figure A7). The mass

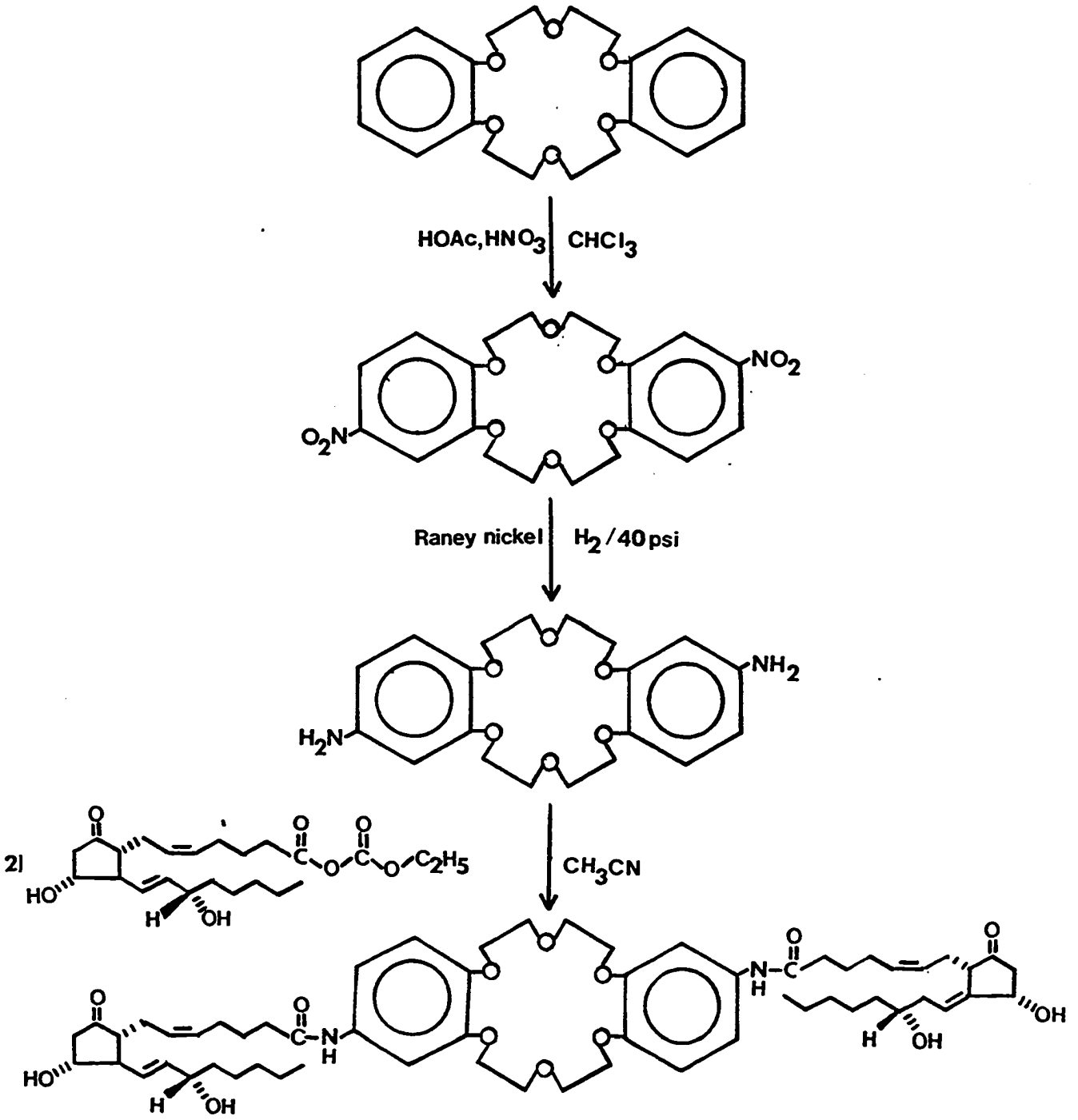


Figure 3. The synthetic scheme for the formation of PGE_2-trans diamide of dibenzo-18-crown-6

spectral analysis was used to confirm the molecular weight. This method was used instead of elemental analysis because a small sample could be analyzed by the mass spectrometer. The fast atom bombardment mass spectrum demonstrated the presence of the molecular ion with a weight of 1058. The melting point of the recrystallized product was 134°C.

Membrane preparation

Polyvinyl chloride membranes were prepared by dissolving 1.0 mg of the PGE_2 trans diamide of dibenzo-18-crown-6 in 5 ml of tetrahydrafuran(THF) followed by the addition of 0.250 ml of dibutyl sebacate. This mixture was then poured into a Petri dish 50 mm in diameter which contained 0.250 g of polyvinyl chloride. The mixture was stirred until the polyvinyl chloride dissolved and the dish was partially covered to allow slow evaporation of the solvent. The result was a flexible membrane, 0.2 mm thick and 50 mm in diameter.

Electrode construction

The antibody electrodes were prepared using a commercially available electrode body (Orion 92-00). This electrode is composed of chemically inert plastic. The cap and the cable are removable so membranes may be installed and filling solution added. Membrane disks (4 mm in diameter) were cut from their polymer sheet. One disk was placed in position and the electrode cap was carefully screwed in place to prevent distortion of the membrane. Once the membrane was installed the filling solution (10^{-2}M KCl) was added to the internal reference chamber and the outer chambers. The cable was then screwed into place and the electrode attached to the potentiometer (Orion 701 A). The electrode was then soaked in 10^{-1}M KCl for several hours prior to use in order to charge the cation binding sites. Each day after use the electrode was placed into this solution where it was stored overnight at 0°C. Figure (4) shows the complete electrode assembly.

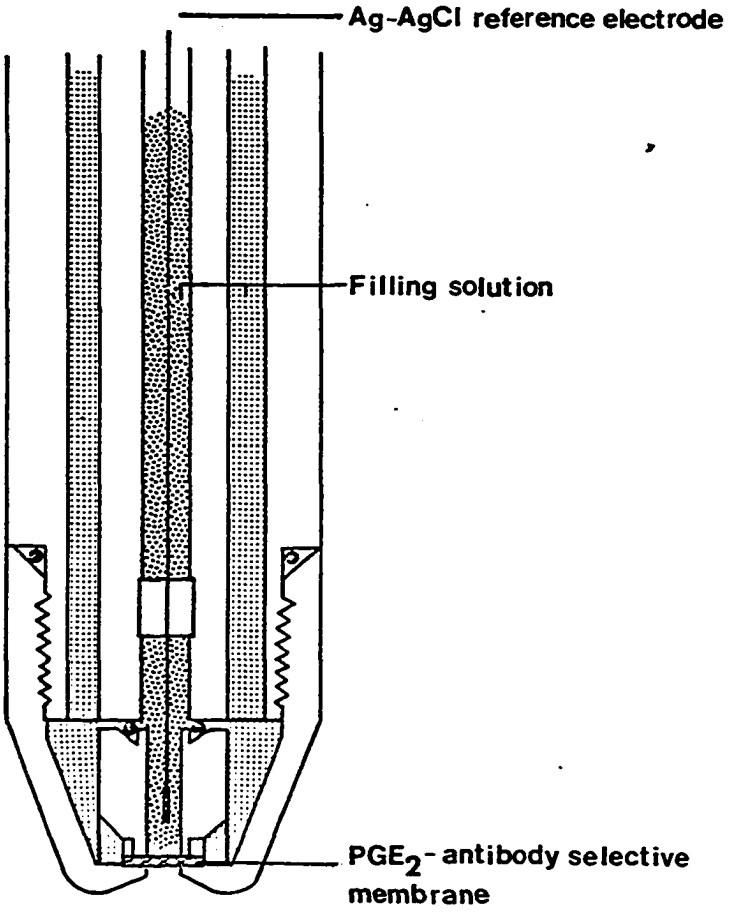


Figure 4. The PGE_2 antibody selective electrode prepared by installing a polyvinyl chloride membrane, composed of 1 mg PGE_2-trans diamide of dibenzo-18-crown-6, 250 mg PVC and 0.250 ml of dibutyl sebacate, into an Orion 92-00 electrode body. The filling solution was 10^{-2} M KCl.

Equipment for testing the electrode

The equipment necessary to perform tests on the electrode included the following: i) reference electrode, double junction with 10^{-2} M NH_4Cl in the outer chamber and the supplied filling solution in the inner chamber, ii) Electrometer, Orion 701 A, a digital meter capable of measuring potentials to 0.1 mV, iii) hot plate-stirrer, Corning PC-351, iv) chart recorder, Linear 2 channel, 100/10 mV full scale, v) constant temperature circulator,Haake FK, vi) 10ml jacketed plexiglass chamber, vii) Faraday cage, constructed with steel fencing material.

The reagents required to test the electrode which were certified ACS grade or better,excluding the sera,included the following: i) anti-PGE_2-antiserum (raised with PGE_2-bovine thyroglobulin(BTG) conjugates in New Zealand white rabbits), lyophilized and reconstituted in buffer ii) nonimmune serum, from New Zealand white rabbits, lyophilized and reconstituted in buffer, iii) potassium cloride (Fisher Scientific Co.,Pittsburgh, PA), iv)calcium chloride(Fisher), v) tris(hydroxymethyl)amminomethane (TRIS) (Sigma), vi) ammonim chloride (Fisher), vii) lithium trichloroacetate

(Orion Research Inc., Cambridge, MA), viii) ammonium nitrate (Fisher),ix) sodium hydroxide (Fisher) x) hydrochloric acid (Fisher) xi) deionized water (Continental Water Systems Inc.).

RESULTS AND DISCUSSION

TESTING

Testing the ion selectivity of PVC membranes containing the PGE_2-trans diamide of dibenzo-18-crown-6

Experiments to determine the ion selectivity of the PVC membrane containing the PGE_2-trans diamide of dibenzo-18-crown-6 were performed. Results from these experiments were used to evaluate the performance of the electrode and to design an appropriate buffer, in which pH and ionic strength could be regulated. The compositions of the filling solutions for the sample and reference electrodes were decided after evaluation of these data. These experiments were divided into two sections. The first evaluated the selectivety of the membrane to potassium, and the second evalated the effects of other ions that might be used to control pH and ionic strength. The other ions tested included; tris(hydroxymethyl)aminomethane, $CaCl_2$, NH_4Cl, and NH_4NO_3.

In the first series of experiments the electrode

containing the ion selective membrane was placed into the bath containing KCl (10^{-1} M) for 12 hours to saturate the cation binding sites. Solutions of KCL (10^{-1} to 10^{-4} M) were prepared by serial dilution. The electrode was immersed in each dilution and the potential was measured in reference to a double junction reference electrode after several minutes of stirring. The data were recorded as E(mV) and plotted vs. the log of the ion concentration. The data were roughly linear, and slopes of the best line as determined by linear regression analysis were calculated. The relationship between potential and potassium concentration was studied at 10, 20, 25 and 30°C.

The effect of tenfold changes in potassium concentration

Transmembrane potential as a function of the potassium gradient was tested with membranes containing PGE_2-trans diamide of dibenzo-18-crown-6 and membranes containing dibenzo-18-crown-6. The gradient was adjusted over the range, log $[K^+]_{in}/[K^+]_{out}$ = 2to (-1). Changes in the gradient were acomplished by altering $[K^+]_{out}$. The data are shown in figures 5 and 6. If these membranes were

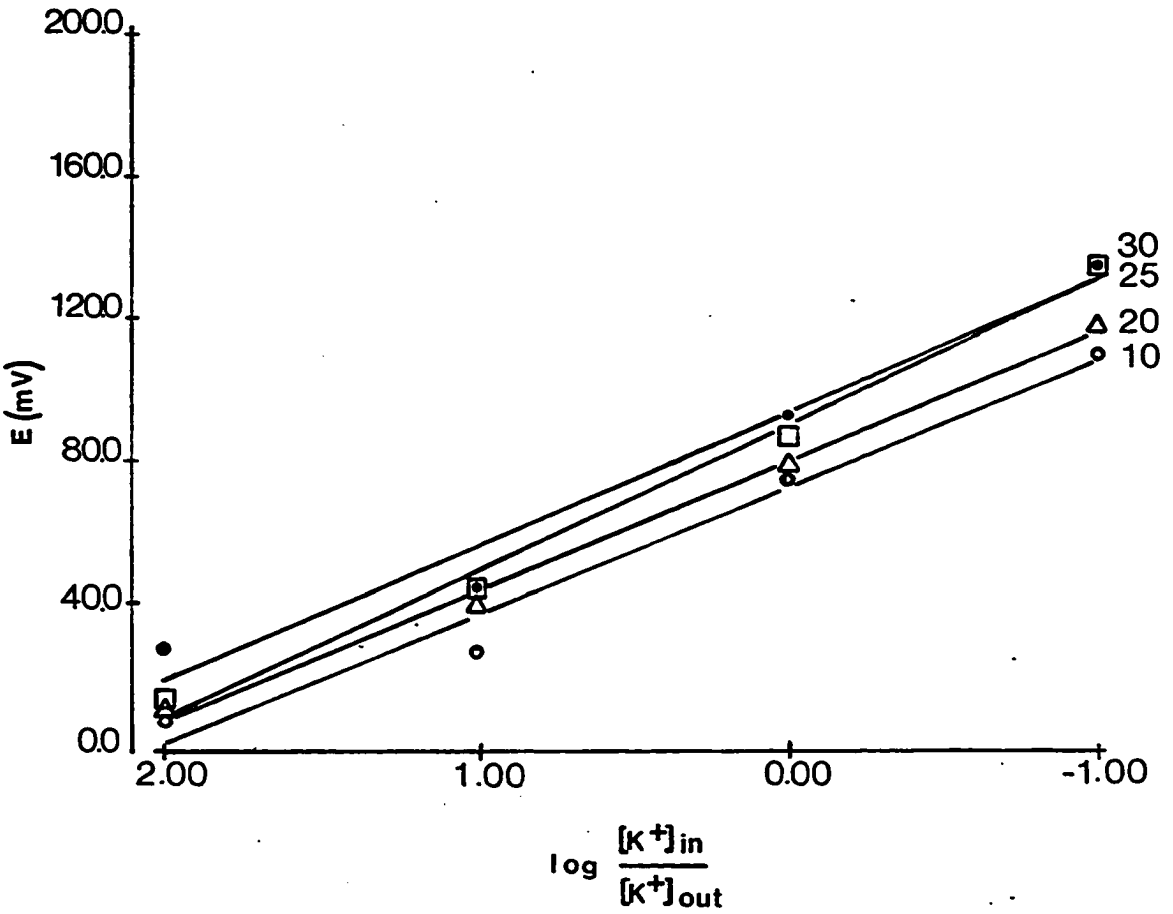


Figure 5. The potentiometric responses of a membrane composed of 1 mg PGE_2-trans diamide of dibenzo-18-crown-6, 250 mg PVC and 0.250 ml dibutyl sebacate plotted against -log of the internal potassium concentration over the external potassium concentration. The internal concentration was 10^{-2} M and the external concentration varied from 10^{-4} to 10^{-1} M. The responses were recorded at 10(o), 20(Δ), 25(□) and 30°C (•). The slopes of the responses are shown in table 1.

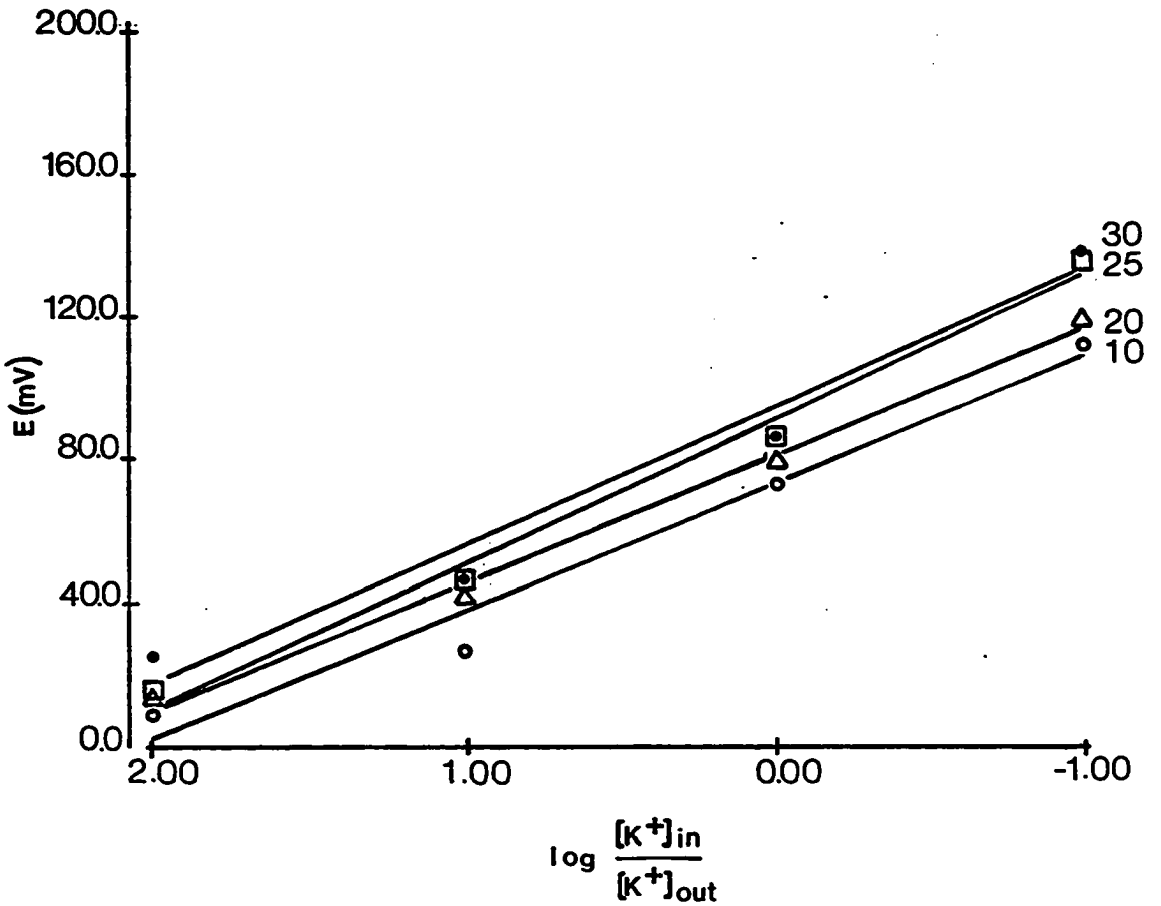


Figure 6. The potentiometric response of a membrane composed of 1 mg dibenzo-18-crown-6, 250 mg PVC and 0.250 ml dibutyl sebacate plotted against the -log of the internal potassium concentration over the external potassium concentration. The internal concentration was 10^{-2} M and the external concentration varied from 10^{-4} to 10^{-1} M. The resposes were recorded at 10(o), 20(Δ), 25(□) and 30(•) °C. The slopes of the responses are shown in table 2.

perfectly selective for K^+, the Nernst equation predicts that the transmembrane potential should change 59.0 mV for each tenfold change in potassium concentration at 25°.

The data show (Tables 1 and 2) the membranes responded to potassium but not in complete agreement with the Nernst equation.

The Nernst Equation:

$$E_m = k - \frac{RT}{ZF} \ln \frac{[X^+]_{in}}{[X^+]_{out}}$$

where, R=gas constant, 8.3143 J $^oK^{-1}$ mol^{-1}

T=temperature in ^oK

Z=ion valance, +1 or +2 for

cations and -1 or -2 for anions

F=Faraday's constant, 9.6487×10^4 C mol^{-1}

at 25°C. the equation reduces to:

$$E_m = k \; -0.059 \log \frac{[K^+]_{in}}{[K^+]_{out}}$$

The actual performance of the electrode containing PGE_2-trans diamide at 25° is listed in Table 3.

TABLE 1

1 mg dibenzo-18-crown-6 membrane vs. potassium

10^{-4}-10^{-1} M

Slope(mV)	Temperature(°C)
35.6	10
35.6	20
40.7	25
38.6	30

TABLE 2

1 mg PGE_2-*trans* diamide of dibenzo-18-crown-6 membrane vs. potassium 10^{-4}-10^{-1} M

Slope(mV)	Temperature(^{o}C)
35.5	10
35.9	20
40.6	25
37.0	30

TABLE 3

PGE_2 electrode vs. K^+ at 25°.

E (mV)	$[K^+]_{out}$	$[K^+]_{in}$
14.2	1.00×10^{-4}	1.00×10^{-2}
45.2	1.00×10^{-3}	1.00×10^{-2}
87.7	1.00×10^{-2}	1.00×10^{-2}
132.5	1.00×10^{-1}	1.00×10^{-2}

The calculated slope for this data is 40.6 mV/tenfold change in $[K^+]_{out}$. The slope itself is not identical to the theoretical values because the membranes were not ideal Nernstian potassium electrodes.

The membrane containing PGE_2-trans diamide of dibenzo-18-crown-6 was tested for response to tris(hydroxymethyl)aminomethane(TRIS), $CaCl_2$, and NH_4Cl.

The data obtained from these experiments are shown in figure(7) and tables(4-6).

TABLE 4

1 mg PGE_2 electrode vs. NH_4Cl at 25°

E (mV)	log $[NH_4^+]$
31.5	-4.000
37.6	-3.000
42.9	-2.000
53.5	-1.000

Slope=7.1

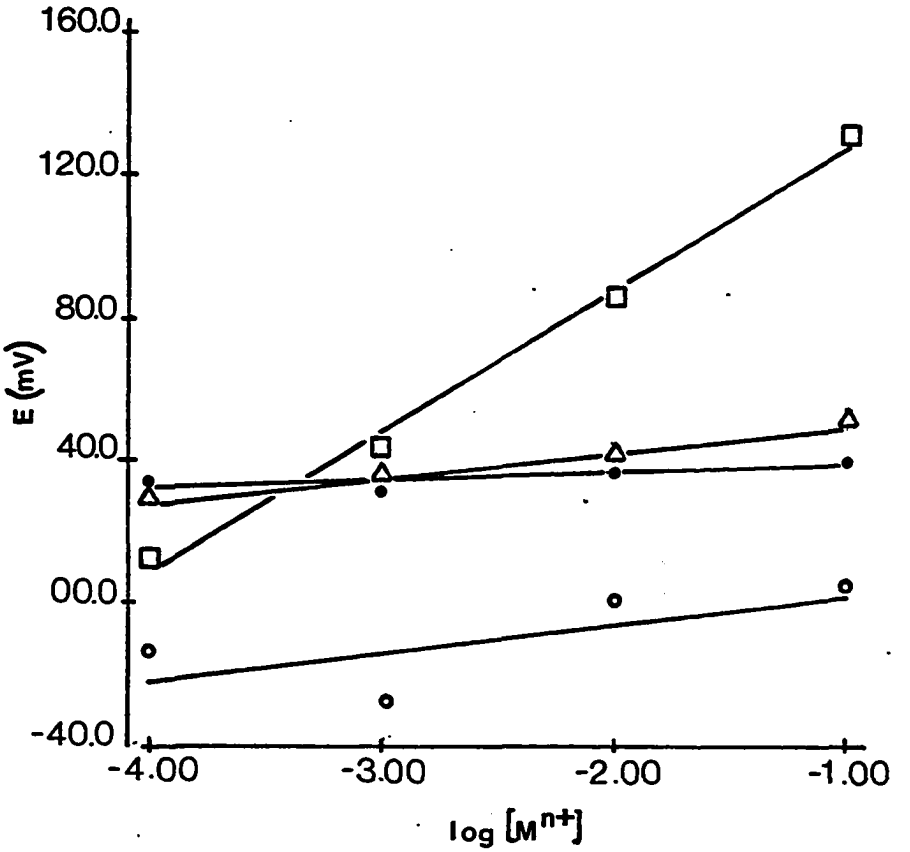


Figure 7. The potentiometric response of a membrane composed of 1 mg PGE_2-trans diamide of dibenzo-18-crown-6, 250 mg PVC and 0.250 ml dibutyl sebacate to KCl(□), NH_4Cl(△), $CaCl_2$(•) and tris(hydroxymethyl)aminomethane(TRIS)(o). The potential was plotted against the log of the external ion concentration which ranged from 10^{-4} to 10^{-1} M. The internal consisted of 10^{-2} M KCl. The temperature was 25°C. The slopes of these responses are shown in tables 1, 4, 5 and 6.

TABLE 5

1 mg PGE_2 electrode vs. $CaCl_2$

E (mV)	log $[Ca^{++}]$
33.9	-4.000
34.8	-3.000
40.2	-2.000
41.2	-1.000

Slope=2.7

TABLE 6

1 mg PGE_2 electrode vs. TRIS

E (mV)	log [TRIS]
-12.6	-4.000
-26.7	-3.000
-1.4	-2.000
7.5	-1.000

Slope=6.3

From these data it is clear that the membrane is more selective for potassium than for the other ions tested. The slope of the potasssium response is much greater than the slopes of the responses to $CaCl_2$ and TRIS. Therefore $CaCl_2$ and TRIS will not contribute sigifigantly to the overall membrane potential if used along with potassium in the buffer. And ammonium chloride is suitable for the use as a filling solution for the reference electrode. The magnitude of the potential for 10^{-4} M NH_4Cl is greater than the potential of 10^{-4} M potassium. To further show that the electrode was not responding to ammonium ions in an effort to explain these elevated potentials the identical experiment was performed with NH_4NO_3 and the data shown in table (7) were collected.

TABLE 7

1mg PGE_2 electrode vs. log $[NH_4NO_3]$

E (mV)	log $[NH_4^+]$
-18.6	-4.000
-8.5	-3.000
1.9	-2.000
22.1	-1.000

Slope=13.3

These data show that the ammonium nitrate solution had lower potential than the ammonium chloride therefore; it is possible that the elevated potential is due to the presence of chloride ions in the test solutions.

From these preliminary ionic studies an experimental buffer was designed which contained: KCL (10^{-3} M), tris(hydroxymethyl)aminomethane (10^{-3} M), and $CaCl_2$ (5.2×10^{-2} M), the ionic strength and pH of the buffer were maintained by this combination at 0.154 M and 7.2 respectively.

The effect of pH on the electrode

To determine the electrodes response to hydrogen ions the following experiment was performed. Samples of buffer were adjusted with dilute HCl or dilute NaOH to pHs ranging from 4-10. The potential of the membrane was recorded after placement of the electrodes in these solutions; each sample was stirred for several minutes until a stable response was achieved. The data was ploted as E (mV) vs. pH. The slope of the data points was calculated by linear regression.

The response of the electrode to pH changes is illustrated in figure (8). The electrode was tested for pH response from pH 4.0-10.0 and the slope of this response was -4.3 mV. The change of potential with change in pH was greatest at low pH.

Experiments planned later in this program included adding antisera to the experimental buffer. Therefore it was necessary to test the effect of serum proteins on pH, because the last study showed that the pH had a substantial effect on membrane potential. The pH of a 4 ml buffer sample at 7.2 was monitored in response to adding

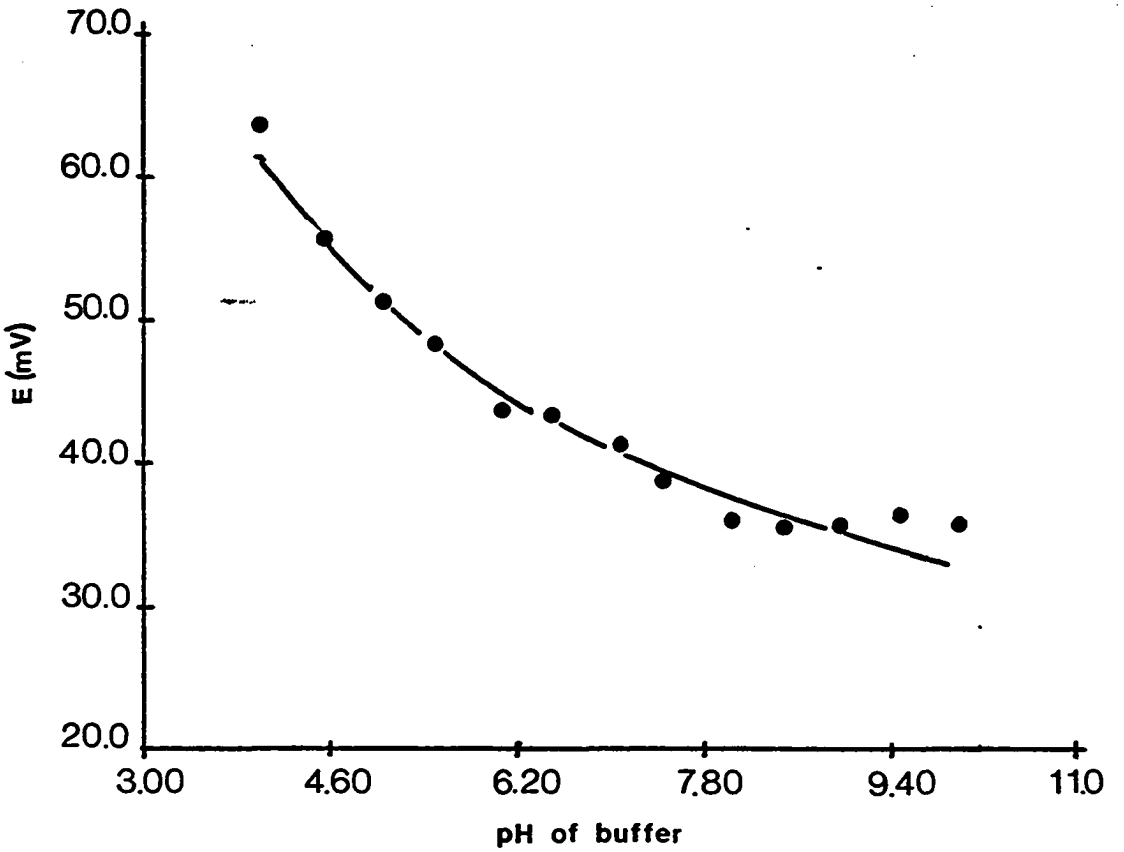


Figure 8. The potentiometric response of a membrane containing 1 mg PGE_2-trans diamide of dibenzo-18-crown-6, 250 mg PVC and 0.250 ml dibutyl sebacate plotted against pH. The internal consisted of 10^{-2} M KCl, the external solution consisted of 10^{-3} M KCl, 10^{-3} M TRIS and 5.2×10^{-2} M $CaCl_2$. The pH of the external solution was varied from pH 4 to 10. The response was measured at 25°C.

reconstituted sera (20-100 µl). This data is shown in figure (9). The pH of the buffer was 7.2 and after the addition of 100 µl of serum the pH was about 7.6.

These data indicate that the serum increases the pH in this range but this increase results in decreased potential response of the electrode. The effects are essentially offsetting, and therefore the pH of the buffer is able to be maintained adequately by 10^{-3} M TRIS. It is likely that a higher concentration of TRIS would have more strigently maintained the pH but the increase may have effected the performance of the electrode; therefore the TRIS concentration was held at 10^{-3} M.

The effect of Anti-PGE_2 antibodies on the electrode

The electrode was tested for response to anti-PGE_2-antibody serum and nonimmune serum. Antibodies were raised in New Zealand white rabbits against bovine thyroglobulin-PGE_2 conjugates (16). The sera were lyophilized and reconstituted in buffer. The concentration of protein in the samples of serum tested ranged from 157.2-786.5 µg/ml (17). The electrode placed

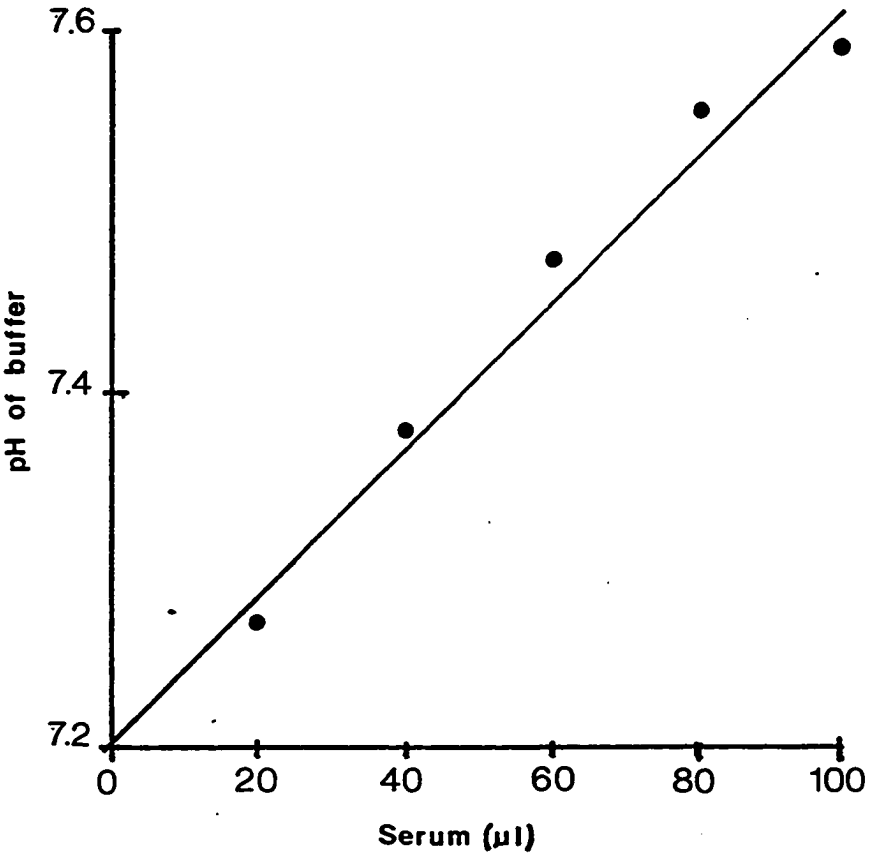


Figure 9. The pH responses of buffer (4 ml) composed of 10^{-3} M KCl, 10^{-3} M TRIS, and 5.2×10^{-2} M $CaCl_2$ at pH 7.2 to the addition of rabbit serum lyopholysed and reconstituted in the same buffer. The pH changes are plotted against the volume of resonstituted serum added (20-100 µl), these volumes correlate with the amount of serum added in later experiments.

in KCl (10^{-1} M) for 12 hours prior to testing. The electrode was then rinsed with buffer and then placed in 4 ml of buffer and allowed to stablize. Reconstituted serum in buffer was added in 20 ul aliquots until 100 µl had been added. After each addition the solution was stirred for several minutes and the electrode response was recorded after stablization. This experiment was performed at 10, 20, 25 and 30°C, and the slope of the line that best fit the data was calculated. These data were plotted as change in E (mV) vs. total protein concentration.

Figure (10) shows the relationship between membrane potential and serum protein concentration at 25°C. The electrode response to anti-PGE_2 antibodies was indicated by an increase in membrane potential after the addition of antisera to buffer. Similar samples of non-immune rabbit serum were added to buffer and the response of the electrode to these samples is shown in the lower plot of figure (10). The addition of nonimmune serum did not increase membrane potential. It is important to note that immune an nonimmune rabbit sera contain equal amounts of protein and ions. The only difference between the two is that immune sera contain anti-PGE_2 antibodies. Therefore,

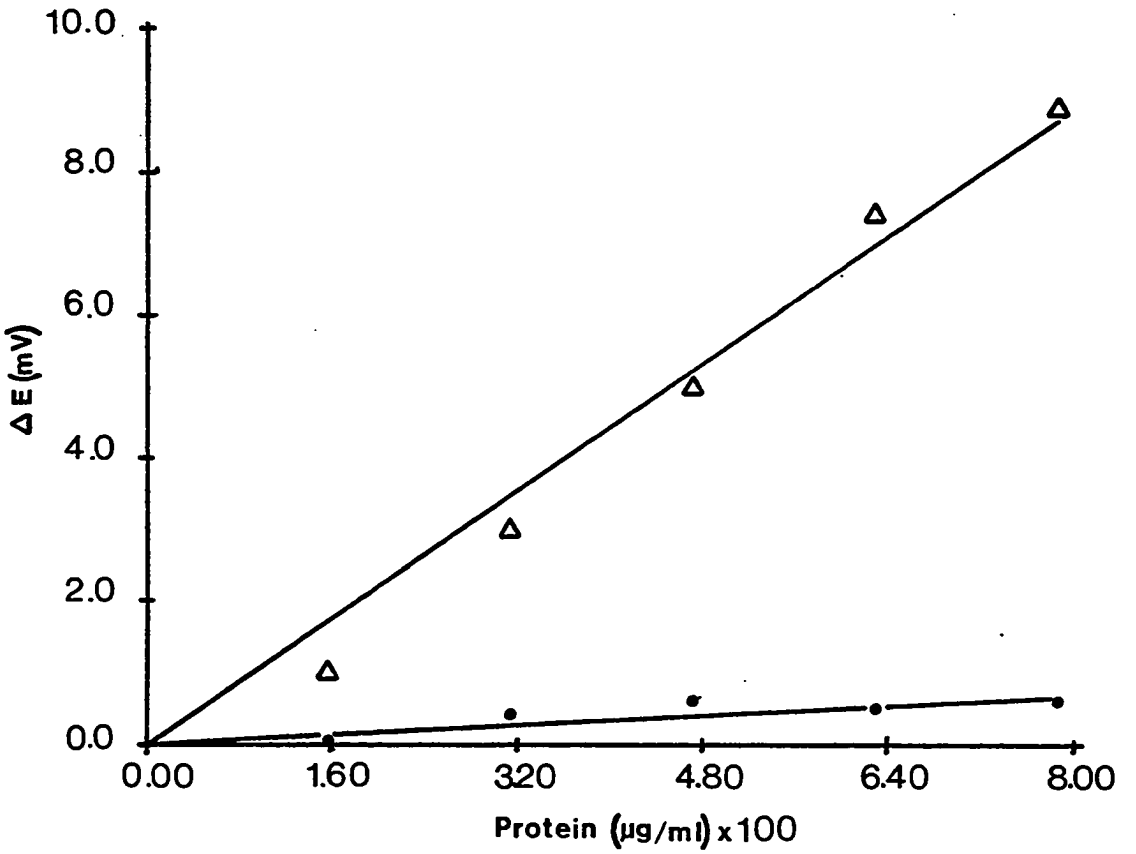


Figure 10. Response of a membrane composed of 1 mg PGE_2-trans diamide of dibenzo-18-crown-6, 250 mg PVC and 0.250 ml dibutyl sebacate to immune, "anti-PGE_2" (Δ), and nonimmune (•), sera. The measurments were made by adding sera reconstituted in buffer, to 4 ml of the same buffer composed of 10^{-3} M KCl, 10^{-3} M TRIS and 5.2×10^{-2} M $CaCl_2$. The membrane potential was increased in a concentration dependent mannor by immune serum. The concentration of antibody is expressed as total protein.

it is likely that the electrode response to immune sera is due to the interaction of anti-PGE_2 antibodies with the PGE_2-trans diamide of dibenzo-18-crown-6 in the membrane.

A membrane composed of 1 mg dibenzo-18-crown-6, 250 mg PVC and 0.250 ml dibutyl sebacate was prepared. This membrane was tested for response to anti-PGE_2 antisera and nonimmune sera following the same procedure used in the above experiment. This experiment was designed to demonstrate the response of the PVC membrane containing PGE_2-trans diamide of dibenzo-18-crown-6 to immune sera was due to a specific interaction of the anti-PGE_2 antibodies with the PGE_2 conjugated ionophore. The electrode did not respond to either type of sera as shown in figure (11). The lack of response of the electrode containing unconjugated ionophore to immune sera further demonstrates that the ionophore(dibenzo-18-crown-6)and the immunogen (PGE_2) together are necessary for the detection of anti-PGE_2 antibodies.

In addition to the tests performed at 25^{o} the response of the electrode to antibody was also measured at 10, 20, and $30^{o}C$. This provided information about the relationship of temperature to antibody response. The slope of the

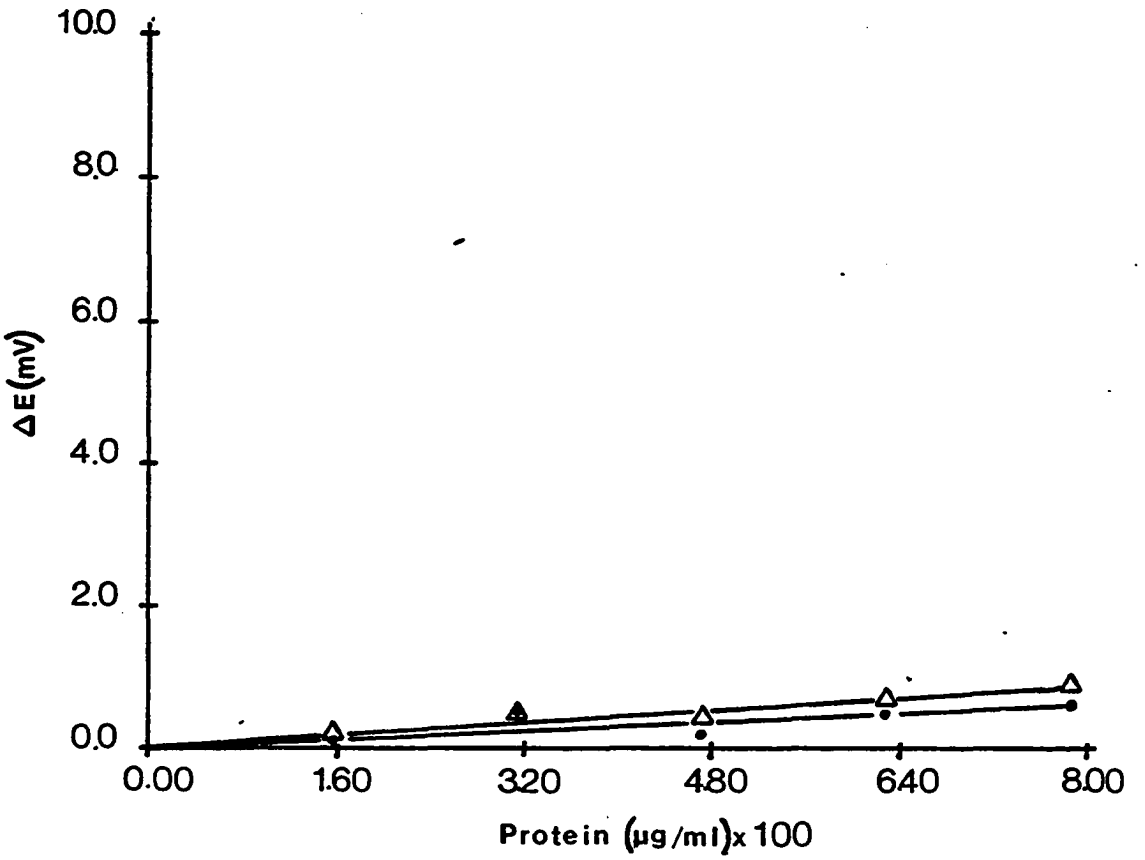


Figure 11. Response of a membrane composed of 1 mg dibenzo-18-crown-6, 250 mg PVC and 0.250 ml dibutyl sebacate to immune, "anti-PGE_2" (Δ), and nonimmune (•), sera. The measurments were made by adding sera reconstituted in buffer, to 4 mls of the same buffer composed of 10^{-3} M KCl, 10^{-3} M TRIS, and 5.2×10^{-2} M $CaCl_2$. The membrane potential was not effected signifigently by either immune or nonimmune sera. The concentration of sera is expressed as total protein concentration.

responses at these temperatures is shown in table (8), the log of the protein concentration was used to determine these data. As indicated in figure (12) the electrode exhibited a temperature dependent antibody response. The greater antibody response at higher temperatures may be due to the increased potassium selectivity of the membrane at higher temperatures, although the highest slope for potassium response was at 25° C. The temperature of 25° was chosen for further experiments because we felt that the response was adequate at this temperature. In later experiments free PGE_2 was added to the buffer and it is more stable 25°C. than at higher temperatures.

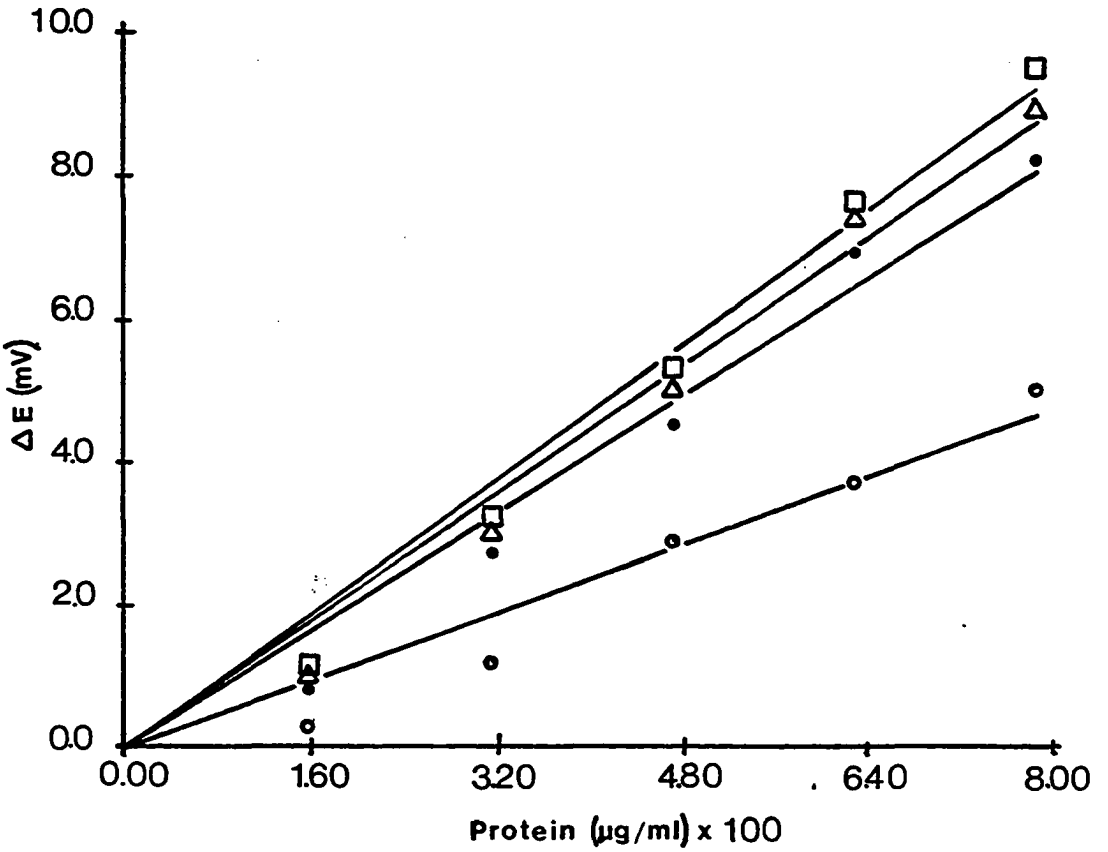


Figure 12. Response of a membrane composed of 1 mg PGE_2-trans diamide of dibenzo-18-crown-6, 250 mg PVC and 0.250 ml dibutyl sebacate to immune, "anti-PGE_2" sera at 10 (o), 20 (•), 25 (Δ), and 30 (□) °C. The measurments were made by adding sera reconstituted in buffer, to 4 mls of the same buffer composed of 10^{-3} M KCl, 10^{-3} M TRIS and 5.2×10^{-2} M $CaCl_2$ at pH 7.2. A temperature dependent increase in antibody response is indicated, the slopes of the responses is shown in table 8.

TABLE 8

Slope of antibody responses at 10,20,25 and 30° C.

Slope	Temperature(° C)
6.63	10
10.62	20
11.29	25
11.82	30

Response time

The antibody effect was fully reversible. The membrane potential increase after the addition of immune sera to buffer was reversed when the electrode was place in buffer containing no sera. The time required to reach 66% maximal response, "on time", of an aliquot containing a concentration of immune serum that appeared on the linear portion of the antibody response curve (471.4 ug/ml) was recorded on chart paper. The wash out, "off time", or the time required for the potential to decrease 66% after the anitbody sample was removed and it was replaced with buffer

only, was also quantitated using the same instrumentation. An example of these data are shown in figure (13). The average on and off times were found to be 7.2±0.3 minutes and 3.6±0.2 minutes respectively. Table (9) shows the data from which the average response times were calculated.

TABLE 9

Electrode response time (minutes)

Trial	on time	off time
1	7.0	3.7
2	7.3	3.7
3	7.0	3.3
4	7.6	3.3
5	7.0	3.7
mean	7.2±0.3	3.6±0.2

The fact that the "off time" is much less that the "on time" follows since the "on time" requires a specific interaction of two molecules. In contrast the disassociation is a more favorable event thermodynamically. The reversibility of the antibody effect is necessary in

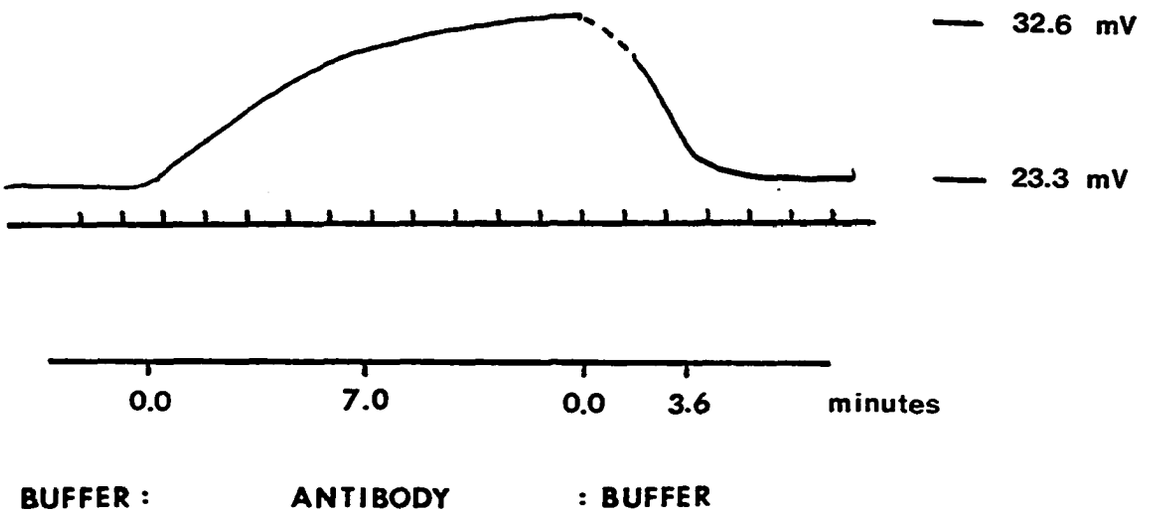


Figure 13. The response time of a membrane composed of 1 mg PGE_2-trans diamide of dibenzo-18-crown-6, 250 mg PVC and 0.250 ml dibutyl sebacate to a 4 ml sample of buffer composed of 10^{-3} M KCl, 10^{-3} M TRIS, and 5.2×10^{-2} M $CaCl_2$ containing immune sera, "anti-PGE_2" 471.4 ug/ml measured as total protein concentration. The "on time" was calculated as the time to 66% total potential response and the "off time" calculated as a 66% decrease from total response. This data illustrates the reversibility of the antibody response. The response time data is summerized in table 9.

order to perform the following experiments with this system.

PGE_2 Challenge

The preceding experiments have illustrated the ability of the PGE_2 antibody electrode to respond to specific antibodies raised to PGE_2. The antibody effect on the membrane in the electrode is fully reversible as demonstrated by the response time studies. The ability of the system to measure antibody can be used in competitive protein binding assay to measure PGE_2 concentration. In competitive protein binding assays a specific protein(antibody) is used which is able to bind a specific molecule. RIA is a competitive protein binding assay. Using labelled compound and specific binding protein unlabelled compound can be measured. This is possible because ideally the labelled and unlabelled compound have equal and high affinity for the binding protein. Therefore a calibration curve can be generated by adding various dilutions of unlabelled compound to samples containing constant amounts of binding protein and labelled compound. The higher the concentration of unlabelled compound the less

bound labelled compound at equilibrium. Using this concept a competitive protein binding assay for PGE_2 was developed using the antibody selective electrode(which responds to anti-PGE_2 antibodies). In this system quantitative determinations are obtained by the potentiometric measurment of unbound antibody rather than radiometric measurment of bound labelled antigen. Anti-PGE_2 antibodies affect membrane bound PGE_2 to produce the response detectable by the electrode. The binding of anti-PGE_2 antibodies with "free" PGE_2 reduces the concentration of unbound antibody and therefore reduces the antibody response in a manner dependent on "free" PGE_2 concentration.

This concept was tested using the following experiment. A solution of buffer containing an average anti-PGE_2-antisera concentration of 471.4 µg/ml was separated into 4 ml samples which were placed in polypropylene test tubes. PGE_2 (0-300 ng/ml) was added to these samples which were incubated for 30 minutes at room temperature. The electrode was placed in antibody solution, containing no PGE_2, and the response recorded. The response of the electrode to this sample of antibody was termed the maximum voltage response (V_o). The electrode was then rinsed

with buffer and immersed in antibody samples containing various PGE_2 concentrations. After stabilization the electrode potential in each solution was recorded. These data were plotted as $\%V_o$ vs. $[PGE_2]$.

The result of this experiment was a PGE_2 concentration dependent decline in antibody response. Increasing PGE_2 concentration decreased the antibody response. The addition of 300 ng/ml PGE_2 to an aliquot of antibody buffer solution completely neutralized the effect of the antibody on the electrode. From these data standard curves were generated over the concentration range of 1-1000 nM PGE_2. A typical assay is shown in figure (14).

Assay Specificity

Experiments to determine the specificity of the PGE_2 competitve protein binding assay were performed using the above procedure with the exception of replacement of PGE_2 with PGD_2 or $PGF_{2\alpha}$.

PGD_2 and $PGF_{2\alpha}$ are both very similar in structure to PGE_2 (figure 15). The results of these studies showed that

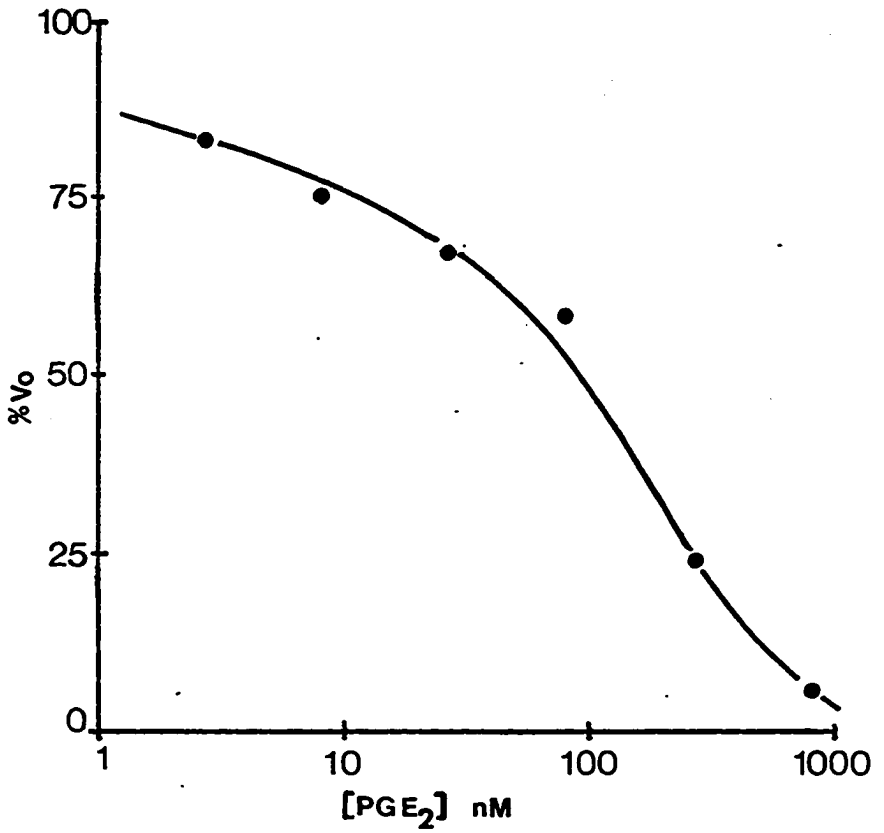
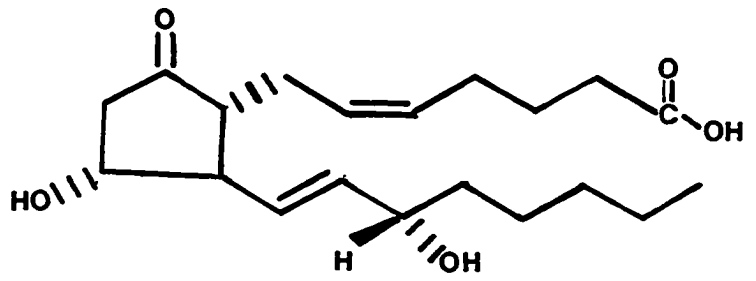
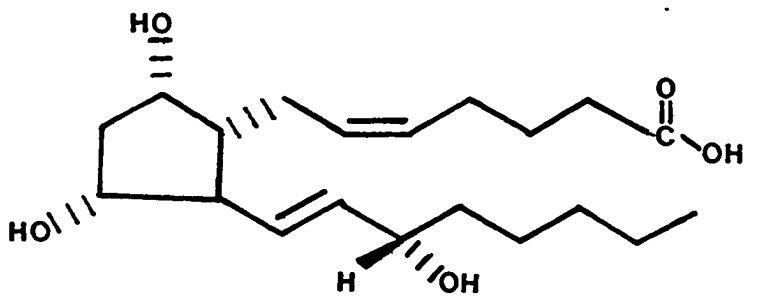


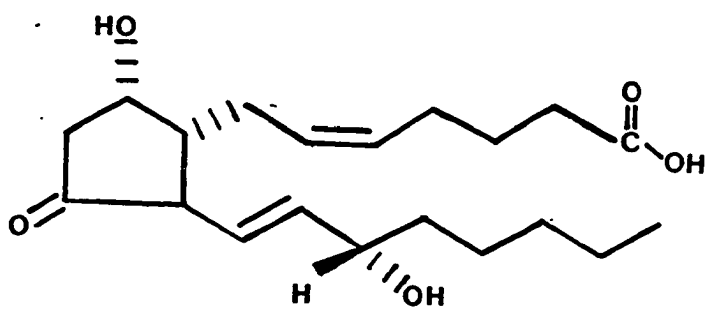
Figure 14. Standard curve for electroimmunoassay. A concentration from the linear portion of figure 10 was selected (471.4 ug/ml). Samples (4 ml) of buffer containing antibody were challenged by the addition of PGE_2 (0-300 ng/ml). The voltage response at this antibody concentration (V_o) was decreased as a function of the concentration of solution-phase PGE_2.



PGE_2



$PGF_{2\alpha}$



PGD_2

Figure 15. The structures of PGE_2, $PGF_{2\alpha}$, and PGD_2.

the assay was specific for PGE_2. The structural similarity of these compounds to PGE_2 made these the most challenging tests of the assay system. The plots of the assays using these compounds are shown in figure(16). PGD_2 decreased the antibody response only slightly at high concentrations. The structure of PGD_2 and PGE_2 are very similar, the only difference being the positions of the carbonyl and hydroxyl groups on the cyclopentane ring are reversed. $PGF_{2\alpha}$ did not decrease the antibody response. This compound is also very close in structure to PGE_2. The difference between the two is the substitution of a hydroxyl group for the carbonyl on the cyclopentane ring in PGE_2. This again demonstrates the high specificity of the antibody for PGE_2.

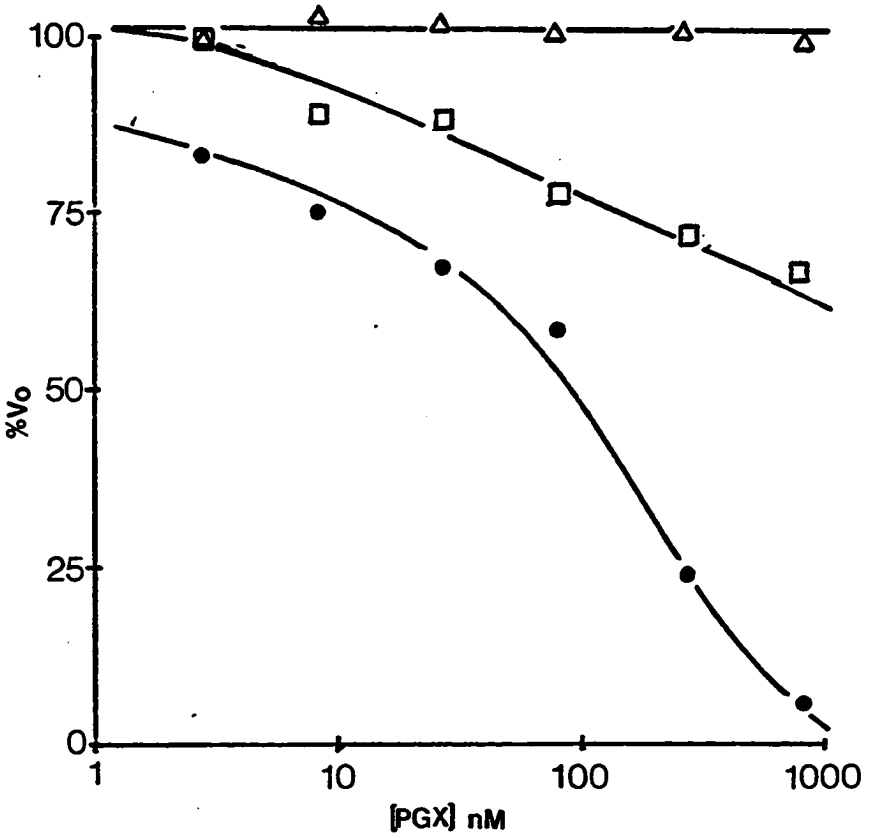


Figure 16. The selectivity of the PGE_2 (●) electro-immunoassay is illustrated by the effect of the substitution of $PGF_{2\alpha}$ (△), and PGD_2 (□) for PGE_2 in the assay.

Conclusions

This study has encompassed several different fields and has brought them together to develop a new analytical system. These fields include synthetic organic chemistry, analytical chemistry, immunology, biochemistry, and physiology. To develop this system the design and synthesis of a conjugated ionophore which would be affected by anti-PGE_2 antibodies was initiated after examination of a similar system developed by Solsky. The conjugated ionophore he developed was sensitive to anti-dinitrophenol antibodies. The structure of this compound included dibenzo-18-crown-6 and dinitrophenol(DNP) covalently coupled. The PGE_2 sensitive conjugated ionophore was composed of PGE_2 molecules coupled to dibenzo-18-crown-6. This conjugate was dissolved in a PVC and cast, forming a membrane. This membrane was then placed into an electrode body so its proporties could be explored. The results of the testing are described in detail in the above pages, briefly the electrode is essentually a potassium selective electrode and this selectivity is enhanced by the interaction of anti-

PGE_2 antibodies with the membrane. The electrode is not a Nernstian potassium electrode. This could be an important characteristic because the antibody may be improving the potassium selectivity which is a possible mechanism of antibody selectivity.

A polyvinyl chloride membrane which showed Nernstian response was described by N. Lakshminarayanaiah (18). This membrane was composed of polyvinyl chloride, diphenyl ether, dibutyl sebacate and valinomycin. This electrode responds to potassium with an average slope of 59.0 mV. The use of valinomycin as the ionophore for the antibody selective electrode was not pursued because the structure of this compound would make attachment of PGE_2 to a site outside the heterocyclic ring difficult. The question raised about this electrode is whether a PGE_2-valinomycin conjugate would respond to anti-PGE_2 antibodies if it was synthesized and placed in a PVC membrane along with the components described. From the information gathered in the study of the PGE_2 antibody sensitive electrode, it is apparent that the valinomycin conjugate electrode would not be a good antibody electrode. It is Nernstian to begin with and therefore the

antibody could not improve on this unless a super-Nernstian response was achieved in the presense of antibody, or unless the antibody could decrease the selectivity of this electrode.

There are several possible mechanisms for the antibody selectivity of the membrane used in this study. The first is that antibody may enhance the selectivity of the membrane for potassium. This mechanism was suggested by Solsky (13), as an explanation for the function of the DNP-antibody selective electrode. These early experiments demonstrated that the ion selectivity of the membrane was affected by the presense of antibody. A shift in selectivity could explain the response of the electrode to antibody. The enhanced potassium selectivity may explain the increased membrane potential which arises when antibody is added to buffer.

Another possible mechanism for the function of the antibody electrode is that the antibody causes the movement of the ionophores to the surface of the membrane thereby increasing the number of potassium binding sites at the surface. This proposed mechanism is dependent upon the mobility of the ionophore in the membrane. The movement of the ionophore although limited is likely because in other

ion selective PVC membranes the selectivity is highly dependent on the type of plasticizer used which influences the fluidity of the membrane.

The binding of antibody to the ionophore may induce changes in the ionophore which increase the binding of potassium. These possible changes include: i) binding of antibody could conceivably cause a conformational change in the ionophore, ii) antibody binding may increase the electron density in the heterocyclic ring of the ionophore, iii) the molecular conformation of the ionophore may change during antibody binding (the structure of the ionophore in the PVC membrane may exist in such a way that the reatively polar PGE_2 molecules attracted by polar interaction may block the heterocyclic ring of the ionophore. Antibody binding may cause the PGE_2 molecules to be pulled away from the heterocyclic ring.).

The antibody also could "carry" potassium ions to the membrane solution interface causing an increase in the actual potassium gradient across the membrane. The antibody, like other serum proteins at pH 7.2 is an acid which can bind potassium ions(19). The antibody is attracted to the surface of the membrane because of the

presence of PGE_2 molecules in the membrane. If the antibody is binding potassium ions and transporting them to the surface of the membrane this would cause an increase in the potassium concentration at the membrane surface which would also explain the increased membrane potential in the presence of antibody.

The PGE_2 electroimmunoassay was possible because the binding of antibody by solution-phase PGE_2 causes a decrease in free antibody concentration. This reduces the electrode response, in a manner dependent on PGE_2 concentration. This system for PGE_2 quantitation has the possibilty of application to monitoring changes in PGE_2 concentration in intercellular fluids, intracellular levels, or on line measurement if the system could be automated. This system also provides a model for other such assays which could be developed for a multitude of antigen-antibody systems.

Other investigations of this system with regard to its function and future possibilities should include the following: i) an experiment designed to determine if potassium is being transported across the membrane which

would make explanations about the mechanism of antibody selectivity possible, ii) the effect of varying membrane antigenic ionophore conjugate concentration on antibody response and range of the PGE_2 assay, iii) the effect of varying antibody concentration in the PGE_2 assay, iv) if the antibody is immobolized in a separate chamber adjacent to the PVC membrane, could PGE_2 concentration be measure directly with this probe, v) the possibility of reversing the system, placing antibody bound to ionophore in the membrane, thereby enabling the measurement of PGE_2 directly.

References

1. S.R. Shrader, Introduction to Mass Spectrometry, (Boston:Allyn and Bacon, 1971), p. 161.

2. Robert Cotter, personal communication.

3. J.C. Giddens, Theory of Chromatography, in E. Heftmann ed.,Chromatography, (New York:Van Nostrand Reinhold, 1975), p. 28.

4. S.H. Ferreira and J.R. Vane, Nature, 216, 868, 1967.

5. J.S. Garvey, N.E. Cremer and D.H. Sussdorf, Methods in Immunology, (Reading, MS:W.A. Benjamin, 1977), pp. 301-306.

6. E.A.Kabat, Basic Principles of Antigen-Antibody Reactions. In eds. H.V. Vunakis and J.J. Langone , Metods in Enzymology, (New York: Academic Press, 1980).

7. J.T. Barret, Texbook of Immunology (St. Louis: Mosby Co., 1978)

8. B. Benacerraf and E.R. Unanue, Texbook of Immunology, (Baltimore:Williams and Wilkins, 1979), p. 6.

9. G. Eisenman, Membranes a Series of Advancements, Vol. 3, Lipid Bilayers and Biological Membranes: Dynamic Properties, (New York:Marcel Decker, 1975), p. 14.

10. G.A. Rechnitz, Science, 214,16,1981.

11. P. Vadgama, Enzyme Electrodes, in A.K. Covington ed., Ion-Selective Electrode Methodology, vol 2., (Boca Raton, Fla.:CRC Press, 1979), p. 23.

12. R.L. Solsky and G.A. Rechnitz, Science, 204, 1308, 1979.

13. R.L. Solsky and G.A. Rechnitz, Anal. Chim. Acta., 123, 135, 1981.

14. M. Bodanszky and M.A. Ondetti, Peptide Synthesis, (New York: Wiley, 1966), p. 88.

15. W.M. Feigenbaum and R.H. Michel, J. Polym. Sci., 9, 817, 1971.

16. K.M. Sanders, The Role of Endogenous Prostaglandin E in the Regulation of Cat intestinal Function, (Ph.D. Dissertation, University of California at Los Angeles 1976).

17. O.H. Lowrey, N.J. Rosebrough, A.L. Farr, and R.J. Randall, J. Biol. Chem., 193, 265, 1951.

18. N. Lakshminarayanaiah, Membrane Electrodes, (New York:Academic Press, 1976), p. 206.

19. H.A. Harper, V.W. Rodwell and P.A. Mayes, Review of Physiological Chemistry, (Los Altos, CA: Lange Medical Publications, 1979), p. 199.

APPENDIX

IR, NMR and MASS SPECTRA

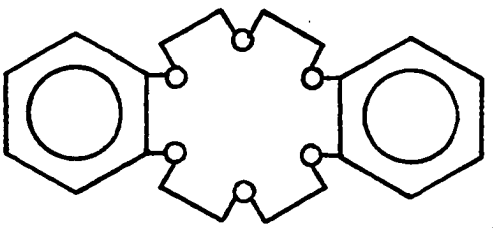
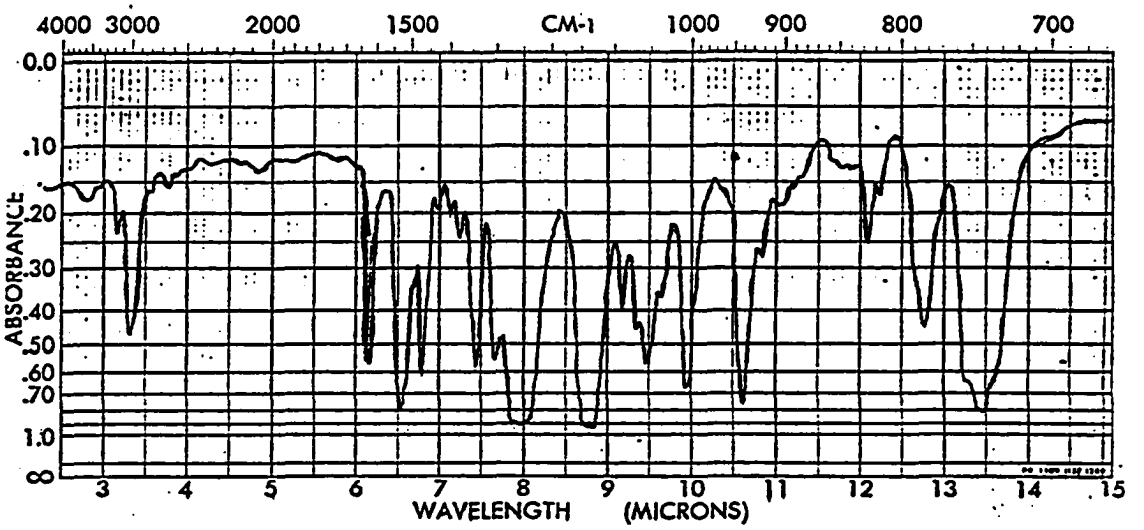
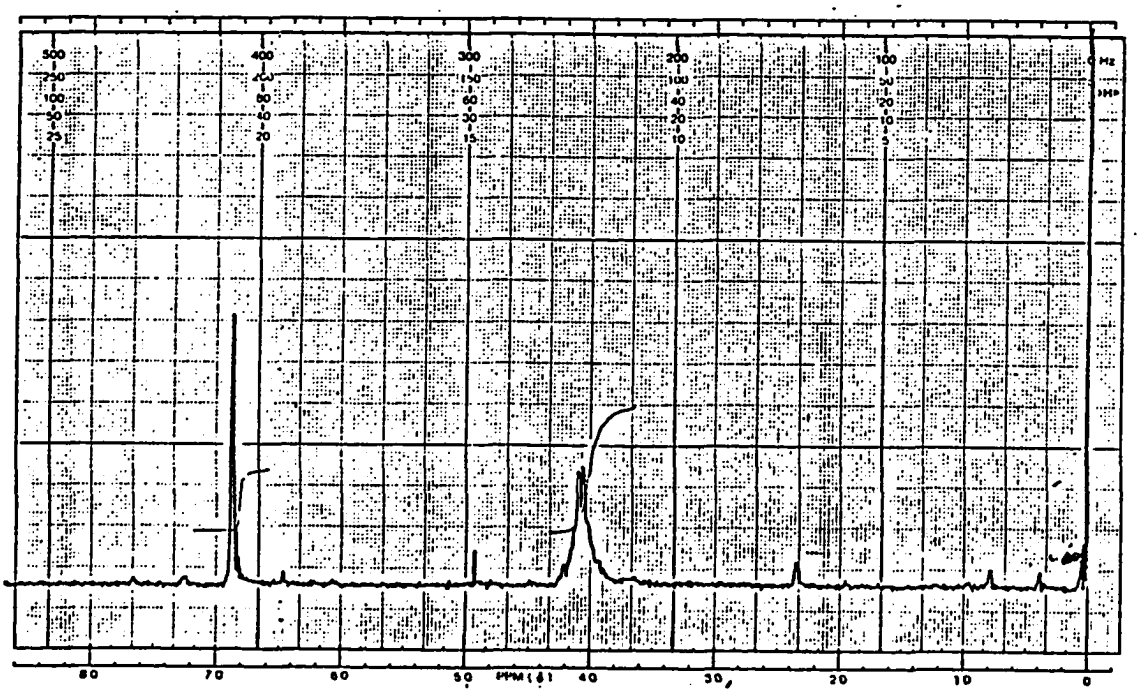


Figure A1. IR and NMR spectra of dibenzo-18-crown-6





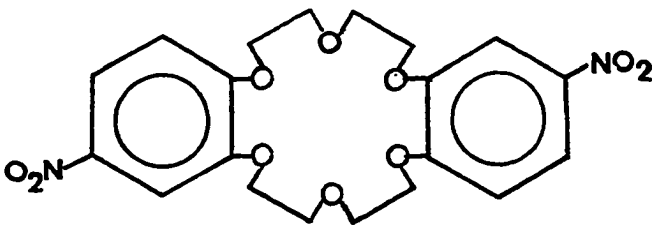
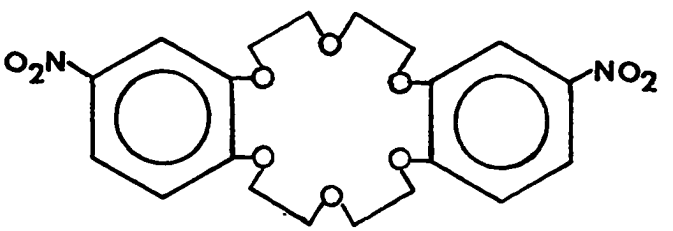
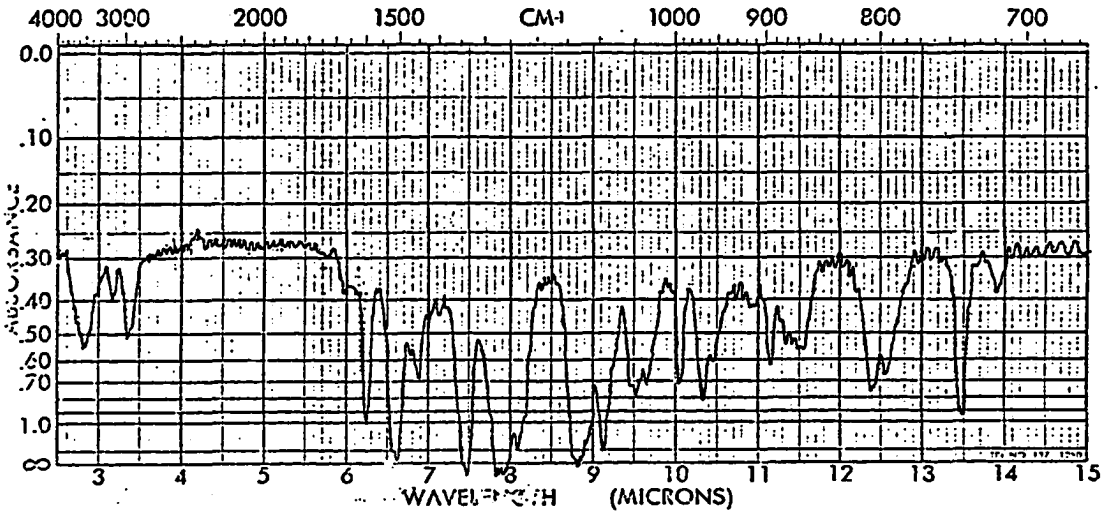
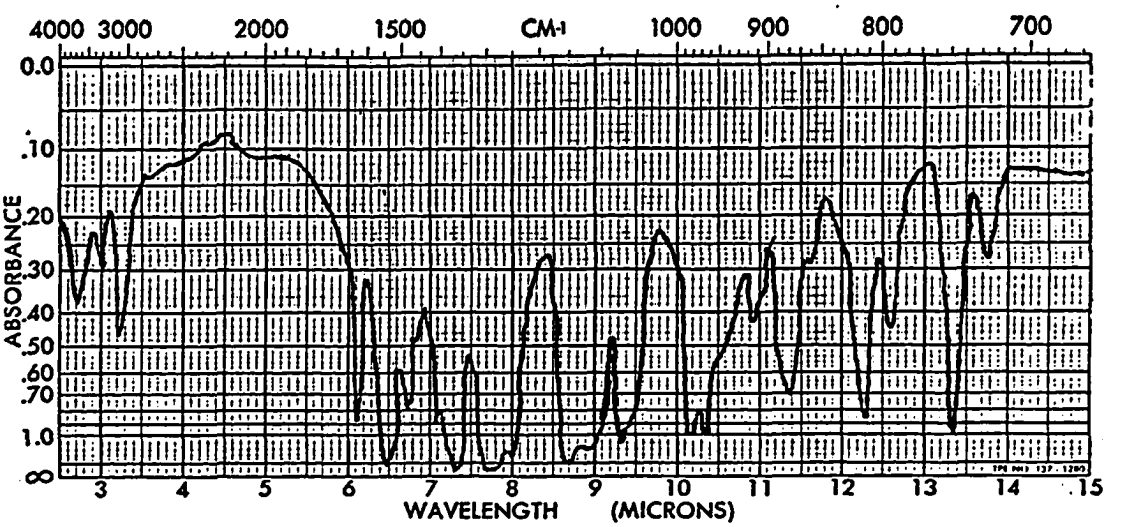


Figure A2. IR spectra of <u>trans</u>-dinitro-dibenzo-18-crown-6 and



<u>cis</u>-dinitro-dibenzo-18-crown-6





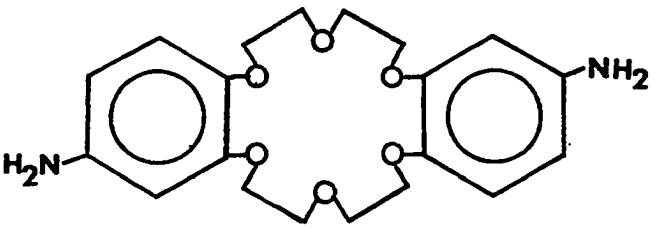
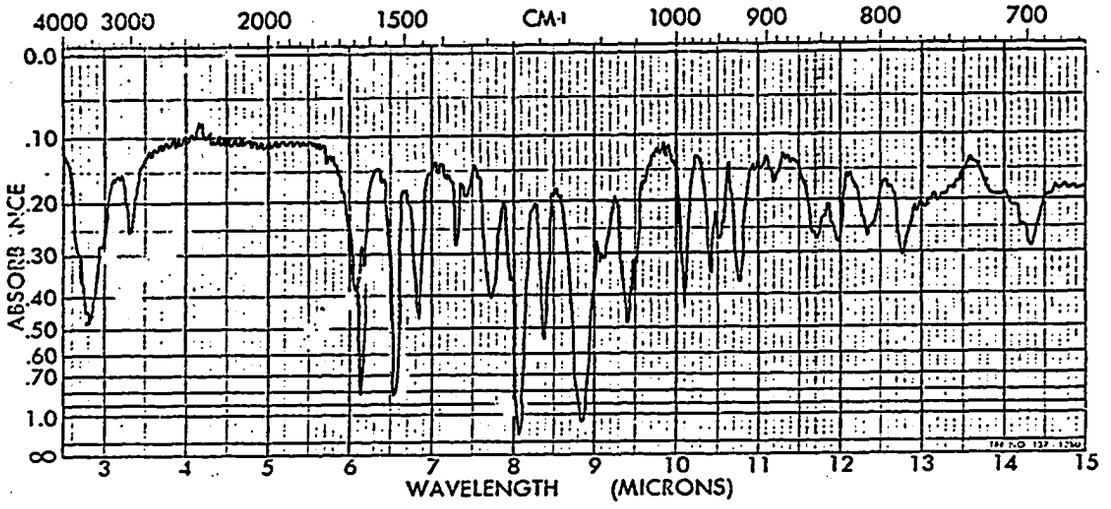


Figure A3. IR spectrum of <u>trans</u>-diamino-dibenzo-18-crown-6



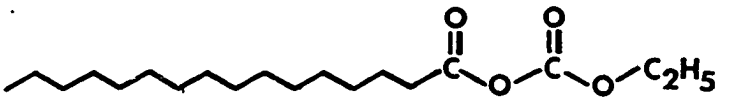
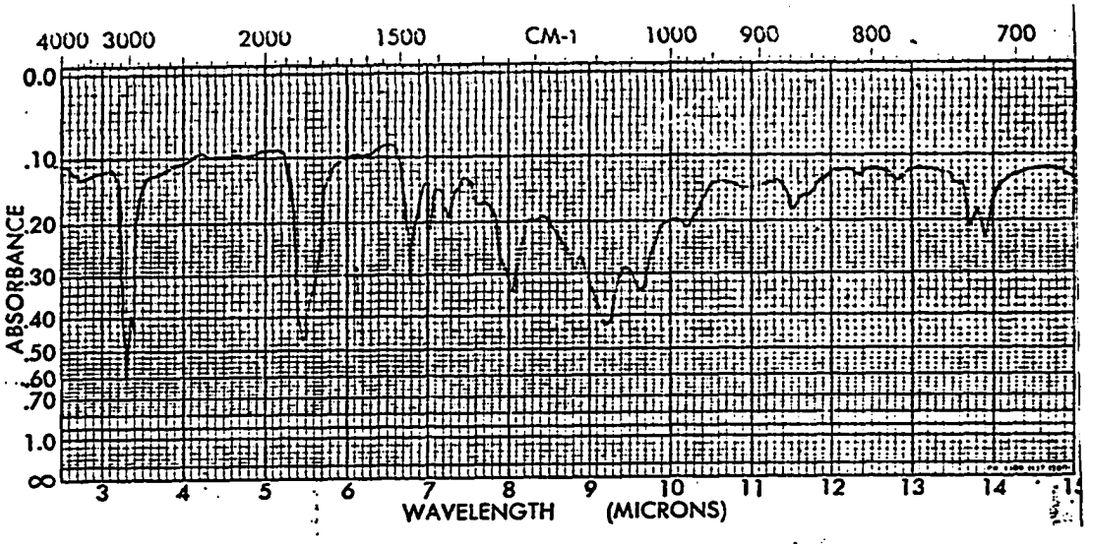
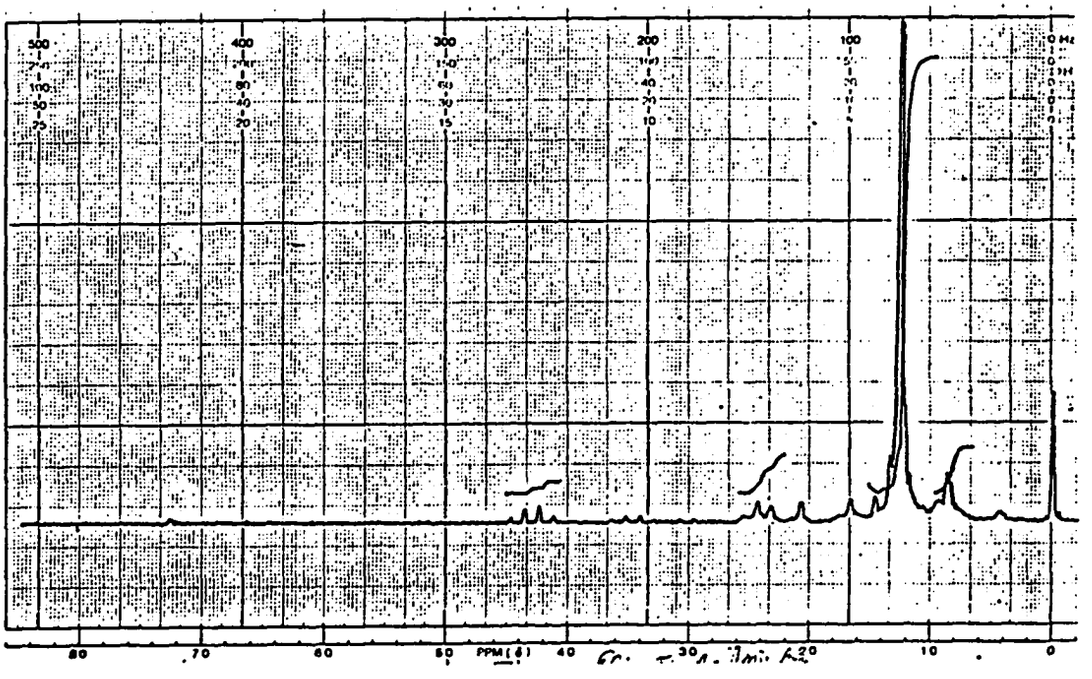


Figure A4. IR and NMR spectra of palmitic acid mixed anhydride





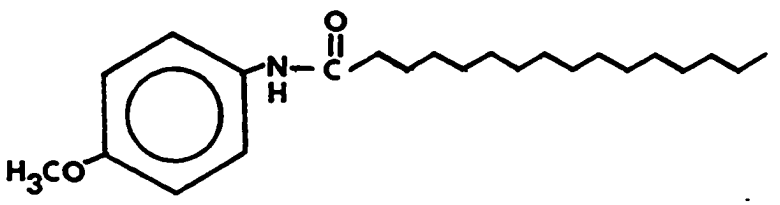
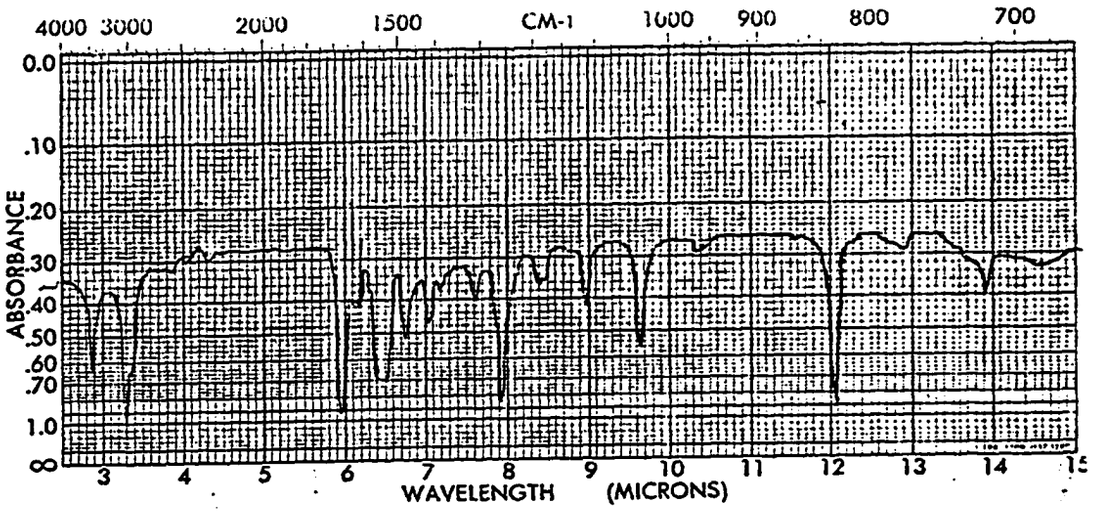
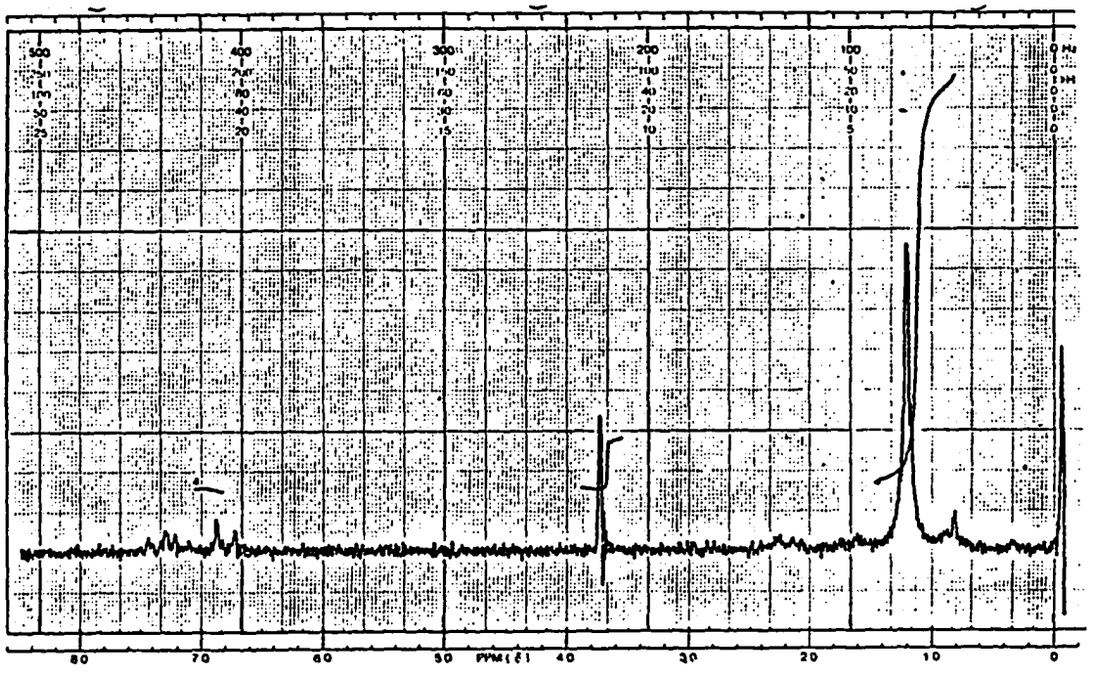


Figure A5. IR and NMR spactra of palmitic acid amide of anisidine





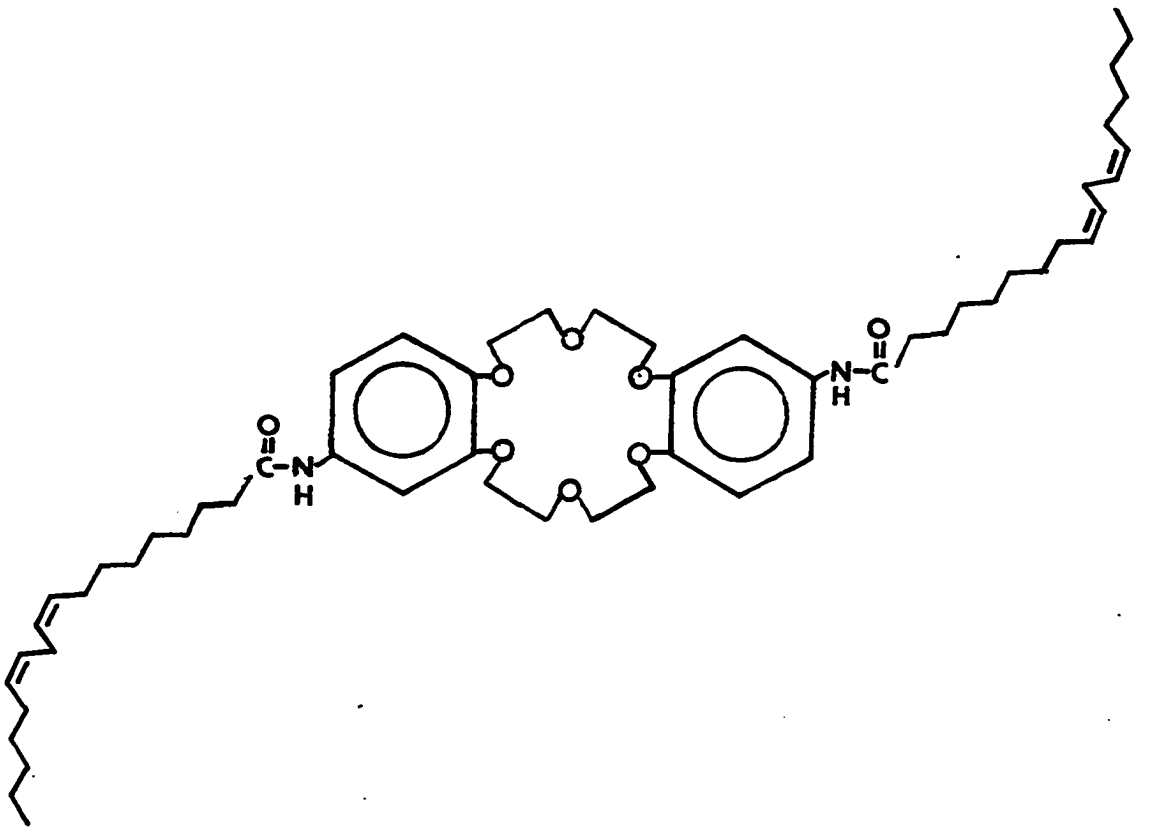
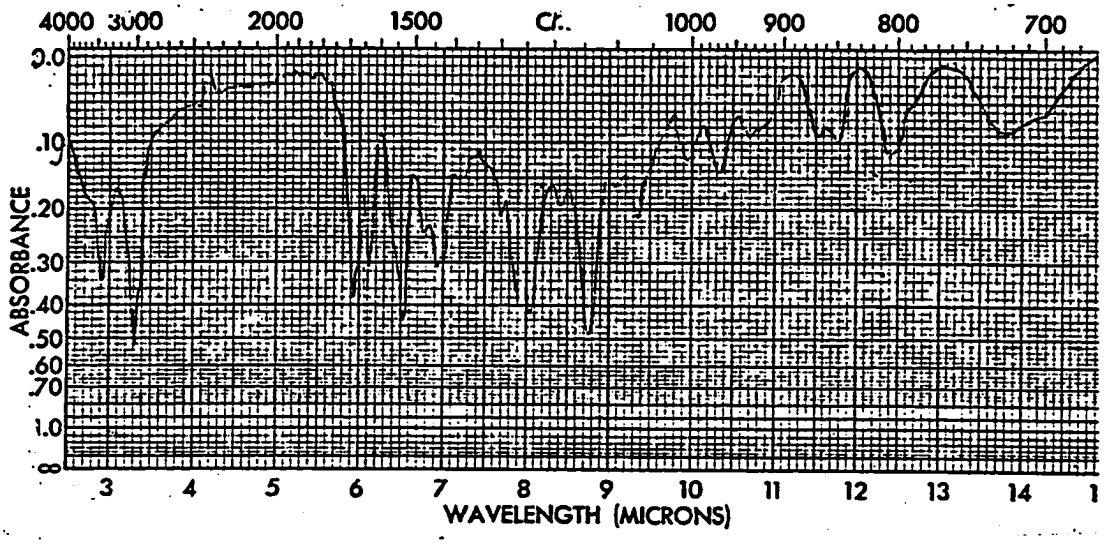
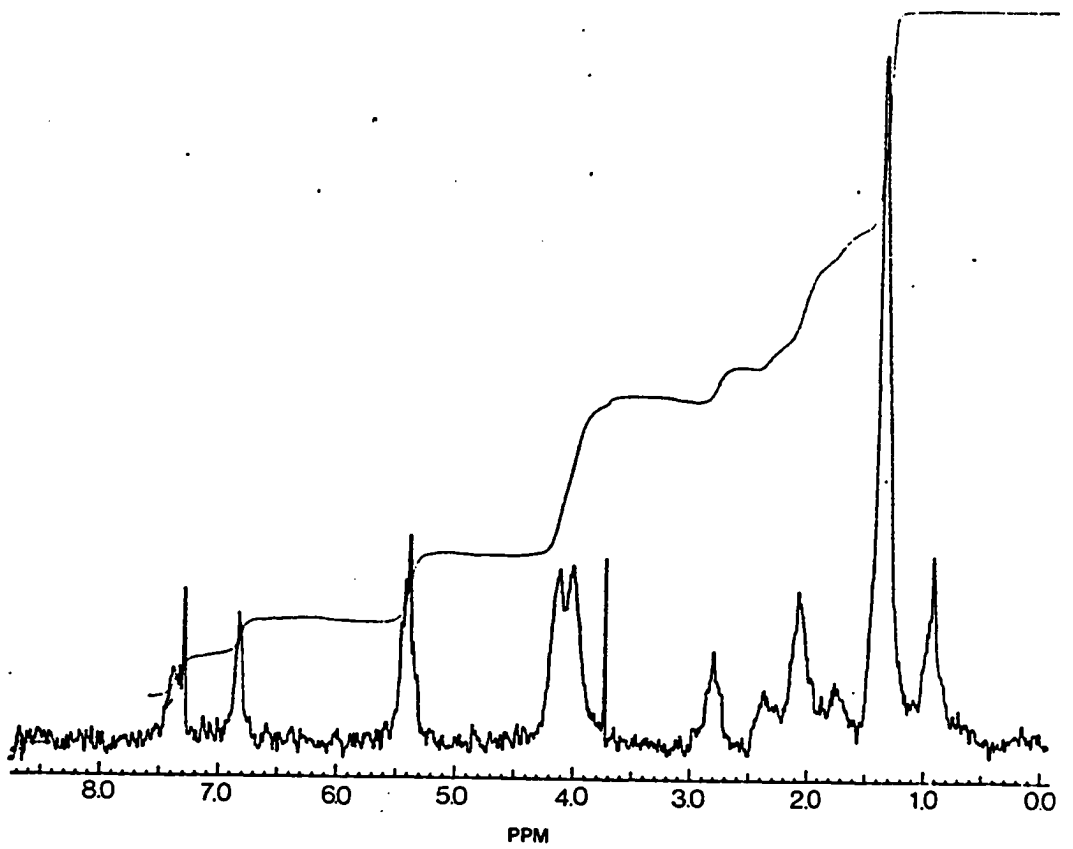


Figure A6. IR and NMR spactra of linoleic acid <u>trans</u>-diamide of dibenzo-18-crown-6





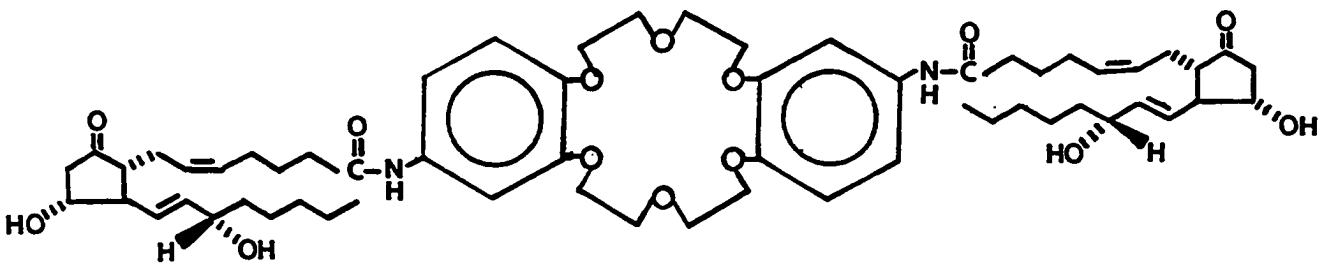


Figure A7. Fast atom bombardment mass spectrum and NMR spectrum of PGE_2-trans diamide of dibenzo-18-crown-6